NY-ESO-1 Re-directed Effector Memory and Central Memory T Cells for the Treatment of Multiple Myeloma

Dissertation zur Erlangung der naturwissenschaftlichen Doktorwürde (Dr. sc. nat.)

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Ich versichere, dass ich die Dissertation selbstständig gefertigt, alle benutzten Quellen und Hilfsmittel angegeben und Zitate im Text kenntlich gemacht habe. Ich versichere ferner, dass ich die Arbeit weder ganz noch teilweise für eine Prüfung an einer Hochschule eingereicht habe.

Zürich, 06.02.2012

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Zusammenfassung

Burnet und Thomas postulierten 1957 die Hypothese, dass Lymphozyten neu entstehende Tumore erkennen und eliminieren können. Erreichen Tumore jedoch eine kritische Masse, entziehen sich diese den verschiedenen Kontrollmechanismen des Immunsystems. Ein möglicher Weg zur immunologischen Tumorkontrolle ist die Herstellung von T-Zellen, in die ein chimärer Antigenrezeptor (CAR) eingebaut wird, der Antigene auf Tumorzellen erkennt. Somit ist es möglich, eine große Anzahl Tumor-spezifischer T-Zellen *ex vivo* herzustellen. CARs sind Fusionsproteine, die aus einem Einzelketten-Antikörper-Fragment (Bindedomäne) und einer intrazellulären Signaldomäne bestehen. CARs der ersten Generation besitzen als intrazelluläre Signaldomäne CD3ζ, wohingegen CARs der zweiten Generation über eine zusätzliche CD28 kostimulatorische Domäne verfügen. Das Einzelketten-Antikörper-Fragment der Bindedomäne erkennt ein Tumor-assoziiertes Antigen, woraufhin die T-Zellen spezifisch Tumorzellen erkennen. NY-ESO-1 ist ein Tumor-assoziiertes Antigen, das in einer Vielzahl unterschiedlichster Krebsarten exprimiert wird. In einem früheren Projekt wurde ein Antikörpermolekül generiert, das das immundominante NY-ESO-1 Peptid 157-165 im Kontext des HLA-A*02:01 Moleküls erkennt.

Um eine Erkennung des NY-ESO-1 Proteins durch autologe $CD8^+$ Zellen zu ermöglichen, wurden in dieser Studie CARs generiert, deren Einzelketten-Antikörper-Fragment das NY-ESO-1 Peptid 157-165 im Kontext des HLA-A*02:01 Moleküls bindet. Entsprechend modifizierte T-Zellen lysierten NY-ESO-$1_{157-165}$/HLA-A*02:01 positive Zielzellen und sezernierten IFNγ in spezifischer Weise. T-Zellen mit Expression eines 2. Generation CARs zeigten sowohl höhere zytolytische Aktivität wie auch Zytokinsekretion im Vergleich zu T-Zellen mit Erstgenerationskontrukt. Phänotypische Analysen CAR tragender T-Zellen zeigten in 40 % der T-Zellen einen ‚Effector-Memory'-Phänotyp. Interessanterweise wurde auch eine ‚Central-Memory'-Population in 3 – 8 % CAR positiver T-Zellen gefunden. Aufgrund der CCR7 Expression wurden Effektor- sowie Gedächtniszellen separiert. Nach Antigen-spezifischer Aktivierung zeigten $CCR7^+$ Gedächtniszellen eine spezifische Herunterregulation ihres CCR7 Rezeptors, welches eine Differenzierung zu Effektorzellen andeutete. Weiterhin sezernierten CCR7 positive Gedächtniszellen vor allem IL-2, ein Zytokin, welches für ‚Central-Memory'-Zellen charakteristisch ist. CAR tragende T-Zellen verhinderten die Entstehung eines Multiplen Myeloms in einem Xenograft Modell. Allerdings verursachten die injizierten T-Zellen nach 30 Tagen eine Xeno-Graft-versus-Host Reaktion.

Diese Ergebnisse stellen eine Grundlage für eine mögliche klinische Anwendung CAR tragender T-Zellen in der Therapie des Multiplen Myeloms dar. CAR tragende T-Zellen, welche den CCR7 Rezeptor exprimieren, sind von besonderem Interesse für die

Weiterentwicklung der adoptiven T-Zelltherapie, um deren Gedächtnisfunktion für eine Langzeitprotektion gegen rezidivierende Tumore zu nutzen.

Abstract

In 1957, Burnet and Thomas hypothesized the concept of cancer immune surveillance which proposes that lymphocytes recognize newly developing transformed cells and eradicate them. However, tumors that are able to form a critical mass escape the various mechanisms of the immune system. One promising approach to immunological tumor control is to genetically modify T cells with a chimeric antigen receptor (CAR) that recognizes antigens on tumor cells. Consequently, it is possible to generate large scale tumor-specific T cell populations *ex vivo*. CARs are single fusion-proteins that contain a single chain fragment (scFv) binding and an intracellular signaling domain. T cells harboring a CAR are called re-directed T cells. First generation CARs consist of an intracellular CD3ζ signaling domain only whereas second generation constructs combine the co-stimulatory CD28 domain and the CD3ζ signaling domain. The binding domain of CARs recognizes a tumor associated antigen which re-directs T cells towards tumor cells. NY-ESO-1 is a tumor associated antigen expressed on a wide variety of malignant neoplasms. In the past, a Fab fragment was generated which recognizes the immuno-dominant peptide 157-165 of NY-ESO-1 complexed in the binding groove of the HLA-A*02:01 molecule.

In this study, chimeric antigen receptors (CARs) were generated consisting of a single chain antibody fragment recognizing the immuno-dominant NY-ESO-1 peptide 157-165 in the context of HLA-A*02:01 linked to the CD3ζ or CD28-CD3ζ activation domain. These re-directed T cells lysed specifically NY-ESO-$1_{157-165}$/HLA-A*02:01 positive cells and secreted IFNγ. T cells expressing second generation constructs were superior to T cells harboring first generation constructs in cytolysis as well as cytokine release. Phenotypical analysis of re-directed T cells revealed an effector-memory phenotype in 40 % of T cells. Interestingly, a subpopulation of central memory T cells was identified comprising 3 – 8 % of the re-directed T cell population. Based on CCR7 cell sorting, effector and memory CAR positive T cells were separated. $CCR7^+$ memory cells demonstrated after antigen specific re-stimulation down-regulation of CCR7 as sign of differentiation towards effector cells and increased secretion of IL-2, the signature cytokine of central memory T cells. Furthermore, CAR re-directed T cells were able to prevent tumor outgrowth of multiple myeloma in a xenograft model but also triggered xeno-GvHD 30 days post injection.

The results are a rational for the potential clinical use of NY-ESO-1 re-directed T cells for therapy of multiple myeloma. $CCR7^+$ re-directed T cells are of special interest for further development of adoptive T cell therapy to not only resolve existing primary tumors but also to keep residual or dormant tumor cells under control.

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Abbreviations

aa	amino acid
ACT	adoptive cell therapy
AI	anti-idiotype
amp	ampicillin
APC	antigen presenting cell
BATDA	2,2':6',2"-terpyridine-6,6"-dicarboxylic acid acetoxymethylester
bp	base pairs
°C	degree Celsius
CAIX	carbonic anhydrase IX
CAR	chimeric antigen receptor
CCR	chemokine receptor
CD	cluster of differentiation
CEA	carcinoembryonic antigen
$C_H1/2/3$	constant heavy chain 1/2/3
CL	constant light chain
CTL	cytotoxic T lymphocyte
Cy	cyanine
DC	dendritic cell
dH_20	deionized water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERBB2	avian erythroblastosis oncogene B 2
EtBr	ethidium bromide
EuTDA	europium 2,2':6',2" - terpyridine-6,6" - dicarboxylic acid
Fab	fragment antigen binding
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanat
FSC	forward scatter
g	centrifugation: earth gravity¸ weight: gram
G	gauge
GIT	gastrointestinal tract
GvHD	graft versus host disease
Gy	gray
H	heavy
h	hour
H&E	hematoxylin and eosin
H_2SO_4	sulphuric acid
H_3PO_4	phosphoric acid
HCl	hydrochloric acid
hIgE	human immunoglobulin E
HLA	human leukocyte antigen
HPV	human papilloma virus
i.p.	intraperitoneal
i.v.	intravenous
IACUC	Institutional Animal Care and Use Committees
ICOS	inducible co-stimulator
IFN	interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motifs
Le^Y	Lewis Y
$MgCl_2$	magnesium chloride
µg	microgram
µl	microliter
µM	micromolar
min	minute
ml	milliliter

mM	millimolar
MM	multiple myeloma
mRNA	messenger ribonucleic acid
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
ng	nanogram
NK	natural killer
NOG	NOD scid IL2Rγ deficient mice
NSG	Nod scid gamma
p	p-value
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	R-phycoerythrin
pmol	picomol
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	incubation: room temperature; PCR: reverse transcriptase
s	second
SAP-1	SRF accessory protein-1
scFv	single chain fragment variable
SEREX	serological analysis of recombinant cDNA expression libraries
SSC	side scatter
T1	type 1
TAA	tumor associated antigen
TAE	TRIS-acetate/EDTA
TAP	transporter for antigen processing
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EFF}	effector T cell
T_{EM}	effector memory T cell
Th	T helper cell
TIL	tumor infiltrating lymphocyte
T_M	memory T cell
TNF	tumor necrosis factor
T_{reg}	regulatory T cells
TRIS	tris-(hydroxymethyl)-aminomethane
U	unit
UV	ultraviolet
V	volume
v/v	volume per volume
VEGFR2	vascular endothelial growth factor receptor 2
w/v	weight per volume
ZAP-70	Z-associated protein of 70 kDa

Abbreviations for nucleotides	
Adenine	A
Cytosine	C
Guanine	G
Thymine	T

Abbreviations for amino acids	
Cysteine	C
Glutamine	Q
Isoleucine	I
Leucin	L
Methionine	M
Serine	S
Threonine	T
Tryptophane	W

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1 Introduction

1.1 The concept of immune surveillance

Proliferation and differentiation of normal cells are governed by many control and feedback mechanisms. However, malignant transformation of cells overcomes these mechanisms and establishes a progressive neoplastic state. Hanahan and Weinberg published in 2000 six common traits, also referred to as hallmarks of cancer that are the underlying principles of the onset and progression of oncogenesis. Cancer cells are self-sufficient in growth signals, insensitive to growth inhibitory signals, resistant to apoptosis, have a limitless replication potential, are able to sustain angiogenesis and invade tissues as well as develop metastasis (Hanahan & Weinberg, 2000). In 2004, Schreiber and colleagues proposed the evasion of immune surveillance as the possible seventh hallmark of cancer (Dunn et al., 2004b; Smyth et al., 2006). This concept has gained more and more acceptance and Hanahan and Weinberg include it as new hallmark in their updated version (Hanahan and Weinberg, 2011).

The concept of immune surveillance conveys that tissues and cells are constantly monitored by the immune system. Thereby, immune cells recognize and eradicate arising tumor cells which prevent formation of a vast majority of newly developing malignant cells (Dunn et al., 2004a). However, malignant cells that are able to form a critical mass escape the various mechanisms of the immune system by either circumventing detection or blocking the cytolytic response (Dunn et al., 2004a).

The importance of immunological surveillance to control cancer development was shown in various pre-clinical and clinical studies. Immunocompromised individuals are more prone to develop certain cancers like Kaposi sarcoma and Hodgkin´s lymphoma. But many of these cancer types are virus-induced (Vajdic and van Leeuwen, 2009). In recent years, increasing evidence is found that the immune system also plays a decisive role in tumor formation and progression in non-virus-induced cancers like multiple myeloma and colorectal cancer (Vajdic and van Leeuwen, 2009). *In vivo* studies showed that genetically engineered mice lacking parts of the innate (natural killer (NK) cells) and/or the adaptive immune system ($CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ Th1 helper T cells) have a higher incidence of cancer occurrence and/or a more rapid tumor growth (Teng et al., 2008; Kim et al., 2007). Furthermore, tumors which arise from immunodeficient mice and subsequently are transplanted to syngeneic hosts are unable to develop secondary tumors. Whereas, cancer cells harvested from immunocompetent mice give rise to secondary tumors in both immunocompromised and immunocompetent hosts (Teng et al.,

2008; Kim et al., 2007). These data further substantiate both the potential host-protective and tumor-editing functions of the immune system during tumor development (Dunn et al., 2004a and 2004b, Dunn et al., 2002). Additionally, clinical studies substantiate the concept of immune surveillance in some forms of human tumors like gastrointestinal, ovarian and head and neck cancers (Bindea et al., 2010; Ferrone and Dranoff, 2010; Nelson, 2008). Pagès et al. and Nelson demonstrated that patients with infiltrations of CTLs and NK cells in colorectal and ovarian cancerous lesions have a better prognosis compared to patients lacking these cells (Pagès et al., 2010; Nelson, 2008). Furthermore, Strauss and Thomas showed in 2010 that immunocompromised organ recipients develop donor-derived cancers which are not detectable in the donors suggesting that the functional immune system of the organ donor is controlling tumor development.

1.2 Tumor associated antigens (TAAs)

Cancer cells need to be recognized by CTLs to be lysed. Thereby, studies revealed that malignant cells express so-called tumor associated antigens (TAAs) which can be used by the immune system to recognize malignant cells.

Technological advances in the field of immunology have led to a more precise understanding and definition of TAAs. Molecules falling into the category of tumor antigens must be produced by tumor cells and recognized by antibodies and/or immune cells. Thereby, some tumor antigens are exclusively tumor specific others are also expressed in normal tissues.

1.2.1 General categorization of TAAs

The above mentioned definition of TAAs and their better molecular characterization have allowed a categorization of tumor antigens into eight groups which are shown in table 1-1 (De Smet et al., 1997; Wang & Rosenberg, 1999; Renkvist et al., 2001; Thompson et al., 2000).

Category of tumor antigen	Description	Example Antigen (Cancer Histology)	Reference
Oncofetal	Typically only expressed in fetal tissues and in somatic tumor cells	CEA (colorectal carcinoma) Immature laminin receptor (renal cell carcinoma)	Shievely and Beatty, 1985 Zelle-Rieser et al., 2001
Oncoviral	Encoded by tumorigenic transforming viruses	HPV E6, E7 (cervical carcinoma)	Breitburd and Coursaget, 1999
Overexpressed/ accumulated	Expressed in normal tissue and in neoplasia, in the latter highly elevated	Her2/neu (breast carcinoma, multiple) SAP-1 (colorectal carcinoma)	Bernard et al., 2002 Seo et al., 1997
Cancer-testis	Expressed in healthy testis and placenta and in tumor cells	NY-ESO-1 (multiple) MAGE-1 (multiple)	Chen et al., 1997a Chomez et al., 2001
Lineage-restricted	Expressed by a single tissue histotype	Melan-A/MART-1 (Melanoma) Prostate Specific Antigen (Prostate carcinoma)	Kawakami et al., 1994 Alexander et al., 1998
Mutated	Genetic mutation or alteration in transcription in cancer cells lead to their expression	ß-catenin (Melanoma, Prostate carcinoma, hepatocellular carcinoma) BRCA1/2 (Breast, ovarian carcinoma)	Robbins et al., 1996 Welcsh and King, 2001
Posttrans-lationally altered	Alterations in glycosylation, etc. that are tumor associated	MUC1 (ductal carcinoma, renal cell carcinoma)	Jerome et al., 1991
Idiotypic	Tumor cells expressing a specific clonotype due to highly polymorphic genes	Ig, TCR (B, T cell leukemia, lymphoma, myeloma)	Bhattacharya-Chatterjee and Foon, 1998

Table 1-1: Categorization of tumor associated antigens.

1.2.2 The cancer testis antigen NY-ESO-1

Since defining the different groups of TAAs progress has been made to assess their value as possible targets for cancer immunotherapy. In 2009, Cheever et al. started a National Cancer Institute pilot project to prioritize cancer antigens in regards to their potential to elicit an immune response. They compared 75 TAAs ranking them by the following criteria: (1) therapeutic function, (2) immunogenicity, (3) role of the antigen in oncogenicity, (4) specificity, (5) expression level and percent of antigen-positive cells, (6)

stem cell expression, (7) number of patients with antigen-positive cancers, (8) number of antigenic epitopes and (9) cellular location of antigen expression (Cheever et al., 2009). NY-ESO-1 from the group of the cancer testis antigens is listed as number 10.

In 1997, Chen et al. discovered the cancer testis antigen NY-ESO-1 using serum from a patient with squamous cell carcinoma of the oesophagus (Chen et al., 1997a; Chen et al., 1997b). The serum was screened against prokaryotically expressed cDNA libraries prepared from tumor specimens for detection of tumor antigens that triggered a humoral immune response with the help of a technology called SEREX (serological analysis of recombinant cDNA expression libraries).

NY-ESO-1 is a protein of 180 amino acids (aa) and a molecular mass of 18 kDa. It is predominantly expressed in the cytoplasm but can be found in the nucleus of some spermatogonia (Jungbluth et al., 2001b). NY-ESO-1 as a potential candidate for immunotherapy is especially attractive because its expression is limited to tumor cells and immune-privileged regions as spermatogonia, oogonia and placenta (Simpson et al., 2005; Jungbluth et al., 2001b) (figure 1-1).

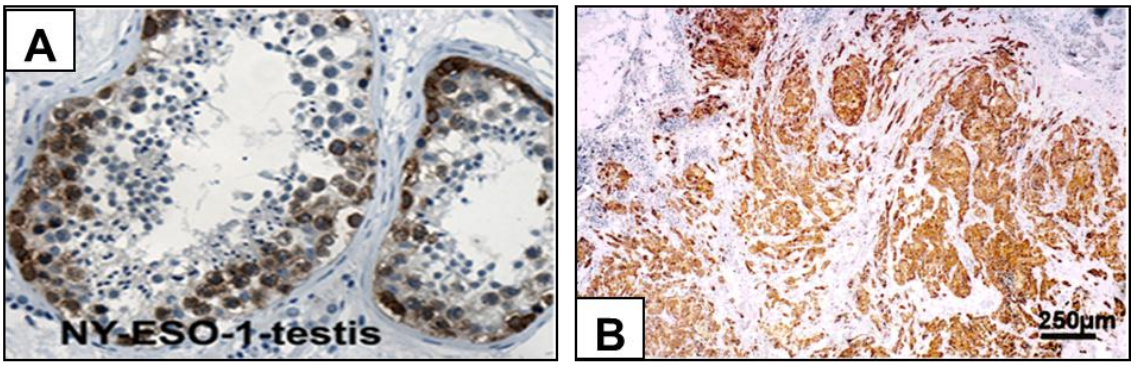

Figure 1-1: Immunohistochemical staining of tissue for NY-ESO-1 expression.

(A) Normal testis shows intratubular staining of spermatogenic cells (adapted from Gjerstorff et al., 2006). (B) Malignant melanoma tissue shows high NY-ESO-1 expression levels (adapted from Jungbluth et al., 2001b).

The function of the NY-ESO-1 protein is not known. There is no rodent homologue and no NY-ESO-1 transgenic animal studies are published (Nicholaou et al., 2006). NY-ESO-1 is expressed in germ cells and trophoblasts but its rapid decline during differentiation processes suggests a role in germ cell self-renewal or differentiation (Jungbluth et al., 2001b; Satie et al., 2002; Takahashi et al., 1995). NY-ESO-1 is expressed in a wide variety of cancer types. Highest frequencies of NY-ESO-1 protein expression are reported for neuroblastoma (82 %; Rodolfo et al., 2003), synovial sarcoma (80 %; Jungbluth et al., 2001a) melanoma (46 %; Barrow et al., 2006) and epithelial ovarian cancer (43%; Odunsi et al., 2003) as detected by immunohistochemistry. 60 % of multiple myelomas after relapse express NY-ESO-1 mRNA as measured with RT-PCR (van Rhee et al., 2005).

Furthermore, accumulation of NY-ESO-1 expression in multiple myeloma has been shown in advanced stages of the disease (Dhodapkar et al., 2003).

NY-ESO-1 is an intracellular antigen. Therefore, it needs to be intracellular processed before it can be presented on the cell surface to the immune system, especially to T cells. Indeed, many T cell responses specific to NY-ESO-1 peptide loaded HLA molecules have been found in cancer patients (Jäger et al., 1998; Gnatjic et al., 2002). Thereby, a wide variety of immunogenic peptides were found complexed to HLA molecules which are recognized by $CD4^+$ and $CD8^+$ T cells. One immunogenic domain of cytotoxic T lymphocytes was discovered in the C-terminal area of the NY-ESO-1 protein compromising of aa 157-165. Furthermore, clinical trials show that CTLs are able to lyse NY-ESO-$1_{157-165}$/HLA-A*02:01 expressing tumor cells after vaccination with NY-ESO-$1_{157-165}$ peptide (Dutoit et al., 2002).

1.3 Human leukocyte antigen (HLA) system

The genes of the HLA system, the major histocompatibility complex in humans are located on chromosome six (Klein, 1986; Klein, 1998; Forbes, 1999). They code for cell surface molecules that present antigenic peptides to T cells and are divided in two classes, class I and class II. The two different classes differ in their structure and function.

Class II molecules are loaded with peptides (at least 13 aa long) from extracellular proteins that previously have been engulfed and processed by an antigen presenting cell (APC). The APC, namely dendritic cells, B cells and macrophages presents the peptide/HLA class II complex to $CD4^+$ T cells, whose main function is to activate/regulate other cells of the immune system.

Class I molecules are expressed on all nucleated cells and on platelets and are loaded with peptides from degraded intracellular proteins. They consist of two non-covalently linked polypeptide chains. The HLA coded α-chain and the β_2- microglobulin subunit are encoded on chromosome 15. The classical genes are HLA-A, B and C. HLA-A*02 is commonly expressed but the allelic variant A*02:01 is the main variant in Northern Asia and Northern America.

1.3.1 Structure of HLA class I molecules

The HLA class I molecule consists of two noncovalently linked polypeptide chains, the larger α chain and the smaller β2-microglobulin. The whole protein consists of four domains; three are formed by the α chain and one by β2-microglobulin. Only the α chain

spans the membrane (Janeway et al., 2001). Proper folding of the α_1 and the α_2 domains leads to formation of a cleft enabling peptide binding (figure 1-2).

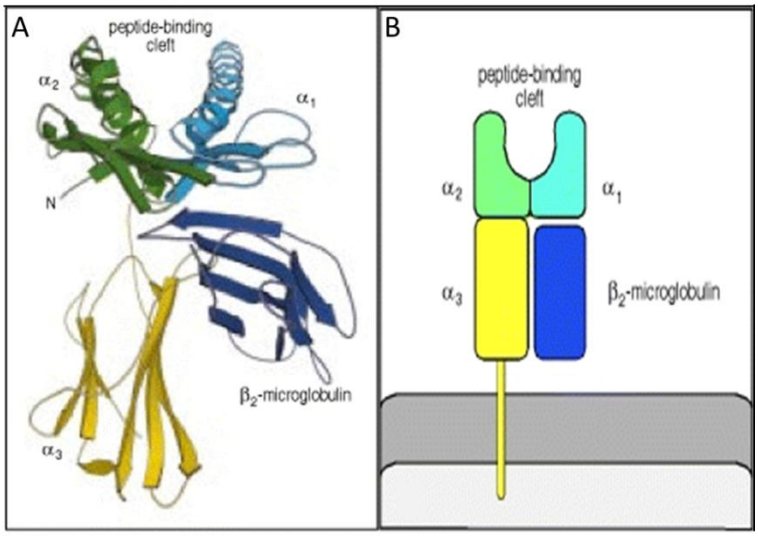

Figure 1-2: The structure of an HLA class I molecule.

Panel A shows a ribbon diagram of the HLA-A2 molecule. Panel B depicts a schematic representation of an HLA class I molecule. Adapted from Janeway et al., 2001.

1.3.2 HLA class I synthesis and peptide loading

Developing an adaptive immune response requires the recognition of an antigen by a corresponding antigen-specific receptor on T cells or B cells. T cells recognize a peptide residing in the binding groove of a HLA-molecule which is presented on the cell surface as a complex. Generation of peptide-HLA-class I complexes is a multi-step process. Peptides loaded on HLA class I molecules can either emerge from self-proteins or from products of viruses or intracellular pathogens. Most HLA class I-associated peptides are 8-11 aa long with a typical length of 9 residues (Falk et al., 1991).

Proteasomes which are abundant and ubiquitous multi-catalytic protease complexes are responsible for the degradation of most proteins and the generation of peptides (Rock et al., 1994). These peptides are between 4 and 20 amino acids in length (Kisselev et al., 1991) which implies that not all peptides can be presented by HLA class I molecules. Extended peptides are further processed by aminopeptidases in the cytoplasm (Reits et al., 2003) or in the endoplasmic reticulum (ER) (Serwold et al., 2002; Saric et al., 2002). Transport of the peptides from the cytoplasm into the ER is mediated by transporter for antigen processing (TAP) (Neefjes et al., 1993). TAP binds to the generated peptides and hydrolyzes one ATP molecule to open its pore for peptide translocations from the cytosol to the lumen of the ER (van Endert et al., 2002) where HLA class I molecules are synthesized. The synthesis and assembly of HLA class I molecules is a complex process in which peptide binding plays a key role. On the cytosolic surface of the ER ribosomes synthesize both poly-peptide chains (α-chain and ß$_2$-microglobulin) of the HLA molecule in a separate step. Newly synthesized HLA class I α-chains bind to the chaperon calnexin.

Binding of β_2-microglobulin to the α-chain leads to dissociation of calnexin from the α:β_2-microglobulin heterodimer. Subsequently, the heterodimer binds to the HLA-class I loading complex, which consists of the molecular chaperones tapasin, calreticulin, Erp 60 (Ortmann et al., 1997) and the TAP complex itself. Tapasin connects the nascent HLA-class I molecule with the TAP transporter for arriving peptides from the cytosol. Stable HLA class I-peptide complexes pass through the Golgi-complex into exocytotic vesicles to the cell surface. These complexes are presented to $CD8^+$ T cells (figure 1-3).

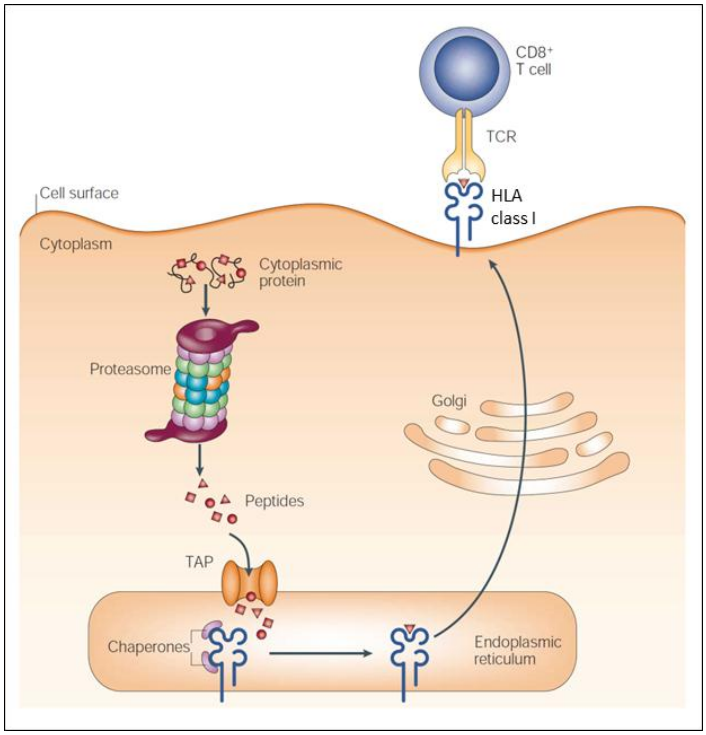

Figure 1-3: HLA class I antigen presentation.
Proteins are degraded to small peptides by the proteasome. The TAP transporter introduces peptides into the ER where they are loaded onto nascent HLA class I molecules. The stable peptide/HLA-complex is transported to the cell surface to be presented to T cells. Adapted from Yewdell et al., 2003.

1.4 T cell activation

The immune system is a complex network of specialized cells and tissues which are able to distinguish self from foreign. Generally, the immune system can be divided in two arms, the innate and the adaptive immune system which is characterized by its ability to recognize already encountered pathogens more efficiently and creates immunological memory. The arm of the adaptive immune response can further be divided in humoral and cellular response, where B cells and T cells are key players, respectively. In general, T cells are comprised of two groups, one regulates the overall complex network of an immune response ($CD4^+$ T cells) and the other is directly cytotoxic and destroys infected cells ($CD8^+$ T cells).

Activation of naïve T cells is dependent on an APC type of the innate immune system, namely dendritic cells (DCs) (Jung et al., 2002; Steinman and Dhodapkar, 2001). Immature DCs capture antigens at the site of infection, process it, subsequently become

activated and travel downstream to the lymph node which drains the infected site. DCs present the processed protein as small peptide fragments complexed to HLA molecules at T cell zones of lymphoid organs to T cells. Naïve T cells continuously migrate from the bloodstream to lymphoid organs where they sample many APCs. When a TCR of a naïve T cell binds to the peptide/HLA complex presented by activated DCs (lock-and-key principle) it stops to migrate and gets activated. The activation of the naïve T cell is at least dependent on three signals: (1) recognition of the antigen through the T cell receptor (TCR); (2) co-stimulation of the T cell through engagement of CD28, CD40, 4-1BB, CD27, ICOS and OX40 and (3) cytokine release such as IL-2 and IFNα (Mescher et al., 2006; Thompson et al., 2006; Xiao et al., 2009). Naïve T cells that receive these three signals will undergo activation, expansion and generate a large antigen-specific effector T cell population.

Signal 1 is provided by the APC expressing the cognate peptide/HLA complex which is recognized by the T cell intrinsic $\alpha\beta$ TCR and the binding of CD8 or CD4 co-receptor to the HLA molecule. Every T cell carries around 30 000 TCRs on its cell membrane which have all the same specificity. Each TCR consists of two polypeptide chains, the TCR α and TCR β chain. Each polypeptide chain compromises a variable and a constant domain, is anchored via a transmembrane domain and ends in a short cytoplasmic tail. The TCR α and TCR β chains are linked via a disulfide bond. The TCR alone is not equipped to transfer the extracellular signal of antigen recognition into the cell. Therefore, each TCR is co-expressed with a non-covalently associated CD3 complex consisting of four polypeptide chains (two ε chains, one δ and one γ chain) as well as a disulfide-linked homodimer of the ζ chain (figure 1-4). These signaling chains are not only necessary for cell surface expression of the TCR but also transport the extracellular signal over the plasma membrane into the cytoplasm. Every CD3 polypeptide chain contains one ITAM motif, every ζ chain three. Phosphorylation of the ITAM motifs is the first intracellular signaling which occurs and leads to further downstream events.

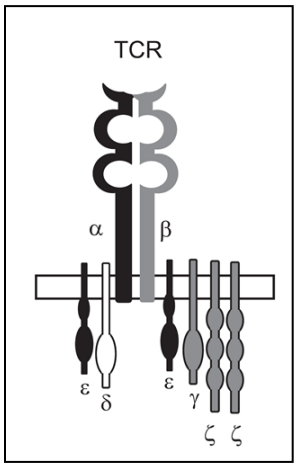

Figure 1-4: Schematic representation of a TCR.

The heterodimeric TCR is co-expressed with the non-covalently attached CD3 complex (ε, δ, γ chains and two ζ chains). Adapted from Dotti et al., 2009.

The second signal originates from APCs which express co-stimulatory molecules on their cell surface. Binding of the peptide/HLA complex in the absence of co-stimulation renders T cells anergic or apoptotic. The best characterized co-stimulatory molecule is CD28 on T cells which is engaged by B7-1 and B7-2 molecules on activated APCs. Binding of CD28 to B7 after interaction of the TCR with the peptide/HLA complex strengthens formation of membrane microdomains and thereby increases TCR proximity to CD3 complexes (Wülfing and Davis, 1998; Viola et al., 1999; Bromley et al., 2001; Wülfing et al., 2002). Receptor clustering also allows even weak TCR ligands to induce successful TCR signaling (Wülfing et al., 2002). After clustering of the TCR complex, the Lck tyrosine kinase which is associated with CD8 or CD4 co-receptors phosphorylates several immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex. Z-associated protein of 70 kDa (ZAP-70) is recruited to the phosphorylated ITAM residues and becomes activated by Lck phosphorylation. Further adaptor proteins are recruited to the activated TCR complex which ultimately leads to the activation of transcription factors through Ras-MAP kinase and calcium- and protein kinase C-mediated signaling pathways. Furthermore, the Lck tyrosine kinase also phosphorylates the sequence motifs of the CD28 intracellular domain which augments TCR signaling. Additionally, CD28 signaling induces transcription factor NFκb to bind to the IL-2 gene promoter which is not activated by sole TCR signaling (Chervin et al., 2009). In summary, CD28 signaling blocks activation of apoptotic pathways, stimulates cell metabolism and induces cytokine secretion.

1.5 Memory T cells

An antigen-specific $CD8^+$ T cell response can be divided into three different phases, namely clonal expansion followed by a contraction phase and subsequent memory formation (figure 1-5 A). During the clonal expansion phase, naïve $CD8^+$ T cells are

activated by recognizing their target on activated DCs and differentiate into effector cells whereby they rapidly down-regulate lymph node homing molecules like CCR7 and CD62L. This allows effector T cells to extravasate into peripheral tissues and reach infected areas (Bromley et al., 2005). Furthermore, effector T cells acquire two critical functions: the ability to lyse target cells and to secrete cytokines (Chowdhury and Lieberman, 2008). Cytotoxicity is mediated through release of perforin and granzymes and/or triggering of Fas. These mechanisms lead to induction of apoptosis of the target cell. Additionally, effector T cells secrete IFNγ and Tumor Necrosis Factor (TNF) after antigen contact, which contribute to increase the local inflammatory response (Slifka and Whitton, 2000). After elimination of the pathogen the antigen-specific effector T cell population contracts due to induction of programmed cell death, whereby 90-95 % of effector T cells are lost. The remaining 5-10 % gradually establish memory populations which can be maintained for very long time. These memory T cells undergo homeostatic proliferation without seeing their antigen for a long period of time depending on the cytokine milieu (Surh and Sprent, 2008). However, they are rapidly reactivated upon renewed antigen encounter resulting in gained cytolytic activity and cytokine secretion. Furthermore, their distribution in various tissues enables them to rapidly protect the host from recurrent infection. In general, memory T cells are subdivided into two main subsets: effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) (Masopust et al., 2001; Sallusto et al., 1999). T_{EM} are CCR7 and CD62L negative and are therefore patrolling non-lymphoid tissues and mucosal sites and possess high cytotoxic potential to immediately confront the pathogen. In contrast, T_{CM} home to secondary lymphoid organs due to CCR7 and CD62L expression without immediate cytotoxicity but they possess superior expansion potential.

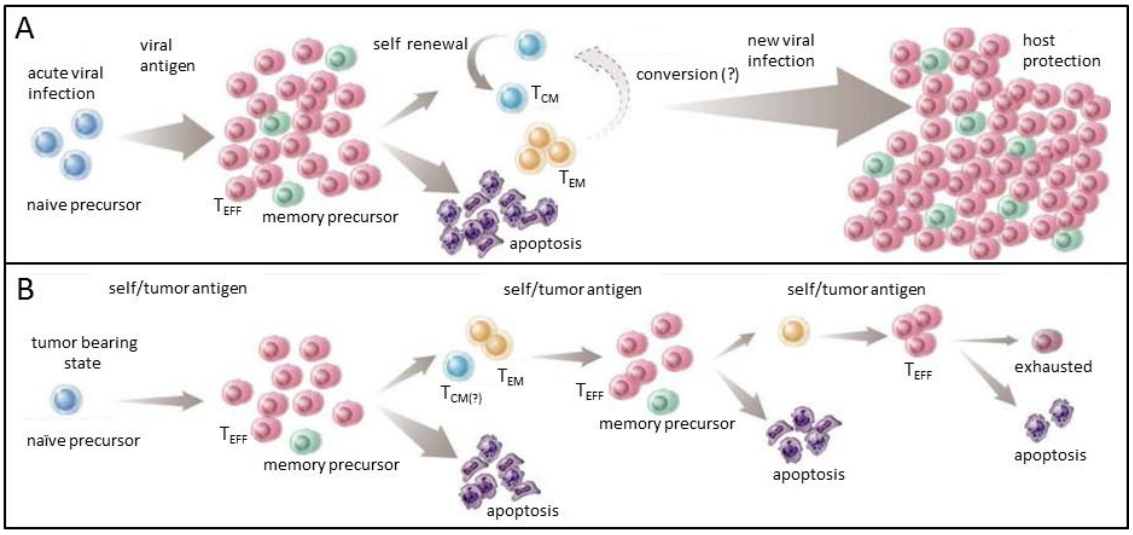

Figure 1-5: Normal (A) and corrupted (B) memory T cell formation.

Panel A shows the formation of memory after an acute viral infection and panel B depicts the same scenario in a tumor-bearing host. In both cases, T cells are activated by their cognate antigen, undergo proliferation and 90 % differentiate to terminally differentiated T_{EFF} cells. The remaining 10 % of cells form a pool of T_{CM} and T_{EM} cells. In the physiological setting (A) the memory pool contributes to further protection of the host whereas, in the tumor-bearing host (B) memory T cells are driven in an exhausted state and are no longer able to control the disease. Adapted from Klebanoff et al., 2006.

An acute infection with a viral antigen can mount an immune response, clear the pathogen, and produce functional memory T cells. However, in the tumor-bearing host this process is altered and/or blocked at different stages (Klebanoff et al., 2006). Especially, the generation of potent memory T cells is highly impaired as depicted in figure 1-5 B. Several mechanisms are identified that contribute to the impaired formation of memory. The induction of T regulatory cells (T_{regs}) by the immunosuppressive environment is shown to negatively impact on the numbers and functionality of memory $CD8^+$ T cells (Kronenberg and Rudensky, 2005; Murakami et al., 2002; Suvas et al., 2003). Furthermore, the presence of other hematological cells, acting as so called cytokine 'sinks' limits the access to homeostatic cytokines as IL-7 and IL-15 which are important for memory formation and maintenance (Gattinoni et al., 2005; Yang et al., 2004). Finally, repetitive/chronic antigen stimulation as it is the case in a tumor-bearing host might lead to functional exhaustion of $CD8^+$ T cells and ultimately drive them into senescence (Klebanoff et al., 2006).

Surface markers were defined for the memory and effector T cell subpopulations to further characterize the pool of $CD8^+$ T cells. Phenotypical characterization of memory and effector T cell subsets has been limited to three or four-color analysis but recent advances in enhancing the resolution of higher order flow cytometry produced a more comprehensive survey. Figure 1-6 depicts the phenotypical changes of naïve T cells on their way to terminally differentiated T cells.

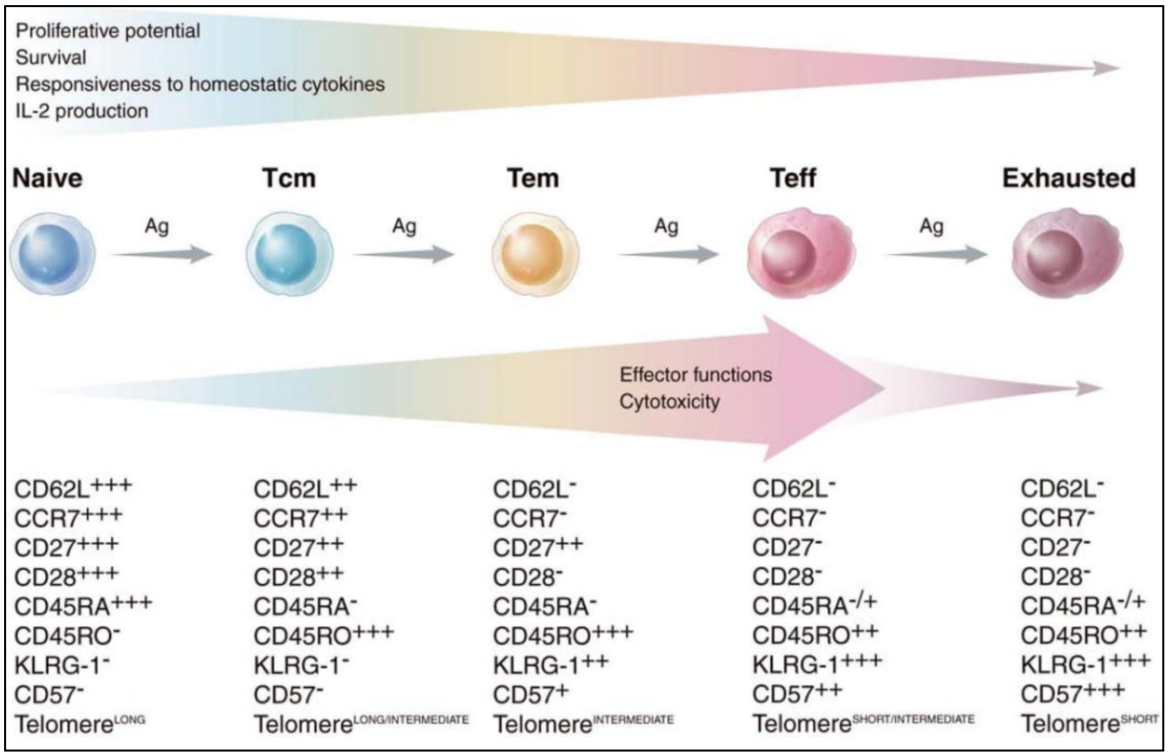

Figure 1-6: Phenotypical and functional changes of the CD8$^+$ T cell compartment induced by chronic antigen stimulation.

Naïve CD8$^+$ T cells become terminally differentiated T_{EFF} and finally enter the state of senescence. The expression levels of the phenotypic markers are described as (+++) high expression (++) intermediate expression and (+) low expression. Adapted from Klebanoff et al., 2006.

Employing flow cytometry with five distinct memory markers, Walker et al. identified in disease-free melanoma patients gp100 specific memory CD8$^+$ T cells (Walker et al., 2008). Thereby, they describe early T_{EM} (CCR7$^-$CD45RA$^-$CD57$^-$CD27$^+$CD28$^+$) and late T_{EM} (CCR7$^-$CD45RA$^-$CD57$^-$CD27$^+$CD28$^-$) as well as T_{CM} phenotypes (CCR7$^+$CD45RA$^-$CD57$^-$CD27$^+$CD28$^+$). Furthermore, T_{EMRA} cells, previously described by Sallusto and Lanzavecchia (Sallusto et al., 1999) by four-color analysis are assigned the following five-color combination CCR7$^-$CD45RA$^+$CD57$^-$CD27$^+$CD28$^{+/-}$. This phenotype designates the re-expression of CD45RA on T_{EM} cells before they acquire CD57 and concurrently lose their CD27 and CD28 molecules which lead to the terminal effector state (Tomiyama et al., 2002; Takata and Takiguchi, 2006). Walker et al. introduced a previously not delineated sub-phenotype which expresses CD45RA together with a central memory phenotype (CCR7$^+$CD45RA$^+$CD57$^-$CD27$^+$CD28$^+$). Therefore, these T cells are termed T_{CMRA} and are discovered to be an antigen-educated memory population as this phenotype is only detected after repetitive vaccination. Finally, fully differentiated effector T cells (CCR7$^-$CD45RA$^+$CD57$^+$CD27$^{+/-}$CD28$^-$) are also found among the tumor-antigen specific subpopulation.

1.6 Chimeric Antigen Receptor (CAR) engineered T cells

The approach of cancer immunotherapy and, in particular, adoptive cell therapy (ACT) is still considered to be in its infacy. Since first reports of adoptive transfer of cytotoxic T cells (Kolb et al., 1990) or tumor infiltrating lymphocytes (TILs) (Rosenberg et al., 1988) laid the groundwork for human application of the treatment of cancer the interest in genetic engineering of T cells to re-direct their effector function has been greatly increased. The basis for genetic T cell engineering was formed by the generation of recombinant DNA in the 1970s and the development of efficient gene transfer methods in the early 1980s. There are two major approaches to re-direct effector T cell specificity, namely TCR- and chimeric antigen receptor (CAR) - engineering.

1.6.1 The concept of adoptive cell therapy

Discovering $CD8^+$ CTLs which recognize presented TAA peptide/ HLA molecule-complexes on the cell surface of tumor cells and their subsequent lysis of these cancer cells has given T cell based immunotherapy of cancer much impetus (Rosenberg 2001). The concept of ACT for cancer therapy was hypothesized after discovering tumor-specific and -reactive T cells in cancer patients (Rosenberg et al., 2008). Isolation, *in vitro* activation, expansion and re-infusion of tumor-specific T cells has been applied in different viral and non-viral related malignancies. In 1988, Rosenberg et al. demonstrated for the first time regression of melanoma lesions in patients by infusing tumor infiltrating lymphocytes (TILs). In these early studies, response rates were modest but improved with the addition of host preconditioning using non-myeloablative chemotherapy (Dudley et al., 2002). Despite these promising results, this therapy is limited to a small number of patients since the isolation and expansion of TILs emerged to be labor-intensive, technically difficult and time consuming. To overcome these limitations new strategies are required to generate tumor-specific T cells. Thereby, genetic modification of T cells is a promising approach in order to generate large T cell populations which are re-directed to recognize tumor cells. Two major routes of re-directing effector T cell specificity evolved over the past years. T cells can be engineered to express exogenous $\alpha\beta$ TCRs or CARs (Morgan et al., 2010a).

1.6.2 TCR engineering and CAR engineering

Although gene transfer of native $\alpha\beta$ TCRs achieved some remarking results in melanoma patients (Morgan et al., 2006), it is affiliated with some concerns. Pairing of the endogenous TCR chain with the transgenic one may result in *de novo* receptor specificities which recognize autologous peptide/MHC complexes and thereby convey

autoimmune reactions (Cartellieri et al., 2010). Furthermore, TCRs have an inherent low affinity for their antigen (Corr et al., 1994) and are well known to exhibit a degree of promiscuity to their peptide/HLA ligands (Mason, 1998). One attempt to overcome these limitations resulted in replacing the variable parts of the TCR α and β chains with single chain fragment variable (scFv) derived from monoclonal antibodies (figure 1-7 A). This led to recognition of antigens in a non-HLA-restricted manner (Becker et al., 1989; Gross et al., 1989; Kuwana et al., 1987). The soon followed discovery of the CD3ζ chain being on its own sufficient for activation of T cells (Irving et al., 1993; Letourneur and Klausner et al., 1991) resulted in the cloning of the first CAR construct, where a scFv was fused to the CD3 ζ chain (Eshhar et al., 1993). Eshhar et al. termed this concept the 'T body approach', nowadays these engineered chimeric receptors are termed CARs (Eshhar et al., 1996).

1.6.3 Properties of chimeric antigen receptors

In general, CARs are composed of a specific-antigen binding domain which is derived from a scFv antibody fragment and fused to a flexible hinge domain (from CD8α or immunoglobulin sequence) (Imai et al., 2004; Zhao et al., 2009) which leads via a transmembrane domain to the endodomain which consists of 1 to 3 cytoplasmic moieties (figure 1-7 B). First generation CAR constructs harbor as endodomain CD3-ε, CD3-γ, CD3-ζ derived from the T cell receptor complex or high-affinity receptor FcεRI (Yun et al., 2000; Abken et al., 2003) which transmit the activation signal into the T cell thereby allowing it to gain effector functions and subsequently lyse target cells. Introduction of a co-stimulatory domain, predominantly as a CD28 ITAM fusion protein (Finney et al., 1998; Krause et al., 1998) led to the generation of second generation CAR constructs. In contrast to the physiological activation of TCR and CD28 receptors, CD28-CD3ζ CARs function as one fusion protein and no recruitment of CD28 into the immunological synapse is necessary to get sufficient T cell activation. Therefore, CD28 co-signaling is provided without the engagement of B7.1 molecules. Third generation constructs comprise one further co-stimulatory moiety primarily from the B7 family (ICOS) or the tumor necrosis factor receptor superfamily (4-1BB, OX40) (Croft 2009; Greenwald et al., 2005). In most cases, the antigen binding domain recognizes a cell surface protein in a HLA-unrestricted manner. To access intracellular antigens scFv were selected which bind in a TCR manner a peptide complexed in a HLA molecule (Stewart-Jones et al. 2009, Willemsen et al., 2005).

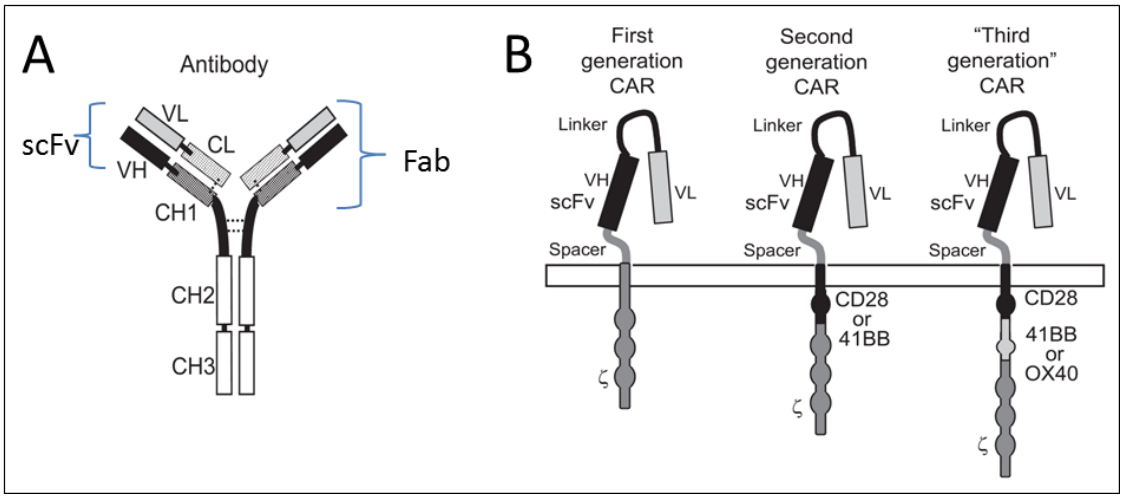

Figure 1-7: Schematic representation of an antibody (A) and of different CAR constructs (B).
(A) The scFv consists of variable heavy chain (VH) and variable light chain (VL) domains, whereas the Fab fragment yields additionally two constant domains (constant light chain (CL) and constant heavy chain 1 (CH1)). (B) First generation CARs harbor only the signaling domain ζ, second generation CARs an additionally co-stimulatory moiety and third generation constructs yield two additionally co-stimulatory domains. Adapted from Dotti et al., 2003.

1.6.4 Methods to express CARs in T cells

Several methods have been used to express chimeric receptors in T cells and are still under evaluation. Most studies use viral vectors that integrate the CAR sequence in the host DNA which is possible due to the high proliferative potential of T cells (Dotti et al., 2009). Thereby, gamma-retroviral vectors have already been tested in several clinical trials (Rosenberg et al., 1990; Bordignon et al., 1995; Heslop et al., 1996, Bonini et al., 1997) and have been proven as a reliable and safe method to re-direct mature T cells. Limitations of retroviral gene transfer include DNA insertion only in dividing cells (Miller et al., 1990), vectors hold limited cargo (Hu and Pathak et al., 2000), DNA insertion in the genome might cause insertional mutagenesis (Hacein-Bey-Abina et al., 2008) and vectors are expensive to produce for clinical applications. Furthermore, expression of the CAR can decline over time, but after T cell activation it increases (Pule et al., 2008). Nonetheless, in 20 years of treating patients with retrovirally transduced T cells no adverse effect related to insertional mutagenesis was reported (Bonini et al., 2003; Brenner and Heslop, 2003). Additionally, Montini et al. showed that retroviral gene-modified T cells do not undergo malignant transformation in RAG1-deficient mice (Montini et al., 2006). A second, widely used viral vector system is based on lentiviruses. The biggest advantage of lentiviral vectors is their ability to transduce minimally proliferating T cells (Naldini et al., 1996; Hu and Pathak, 2000). Moreover, they have a higher cargo potential, are not as susceptible to gene silencing and have a lower risk to insert in `hot spot´ regions which might further reduce the already low risk of malignant transformation

of the cell (Montini et al., 2006). However, safety and efficacy of lentiviral vectors are still under investigation. Recent advances are also made in the field of integrating non-viral vectors including DNA plasmid-based (Park et al., 2007; Till et al., 2008) and transposon-based (Huang et al., 2008; Wilson et al., 2007) gene delivery methods. Further investigation of these systems will show their feasibility. Other approaches include messenger RNA (Mitchell et al., 2008; Zhao et al., 2010, Barret et al., 2011) or protein transfer. The transient nature of the transgene expression makes this a safe approach but its clinical value will have to be explored in future clinical trials.

1.6.5 Phase I clinical studies employing first generation CARs

Based on the antitumor effect of CAR engineered T cells in *in vitro* and *in vivo* models, first phase I clinical studies utilizing first generation CARs have recently been performed and completed. In one study, re-directed T cells were targeted against the ovarian cancer-associated α-folate receptor. Engineered T cells disappeared rapidly after adoptive T cell transfer with barely detectable levels one month after treatment and they showed no biological effect (Kershaw et al., 2006). Another study observed no clinical responses in patients with metastatic renal cell carcinoma treated with re-directed T cells recognizing carbonic anhydrase IX (CAIX). Furthermore, patients developed severe liver toxicities due to unexpected on-target effects against CAIX positive epithelial cells in the bile duct (Lamers et al., 2006; Lamers et al., 2007). The clinical studies mentioned above used retroviral vectors as gene delivery system, whereas Park et al. transferred electroporated CAR positive T cells directed against L1-adhesion molecule specifically expressed in neuroblastomas. In this study, one of six patients showed a partial response (Park et al., 2007). Furthermore, Till et al. conducted a clinical study with electroporated T cells modified to recognize CD20, an antigen present on B cell lymphoma and mantle cell lymphoma. Although administration of low dose IL-2 prolonged persistence of modified T cells, the number of circulating $CD20^+$ B cells did not decrease and antitumor effects were marginal (Till et al., 2008). A promising application of gene therapy was reported by Pule et al. after treating neuroblastoma patients with re-directed T cells recognizing diasialoganglioside GD2. Pule et al. engineered two different T cell populations: virus-specific CTLs and bulk CTLs. Infusion of CAR engineered virus-specific T cells was safe and resulted in tumor regression or necrosis in half of the treated patients (Pule et al., 2008).

Although, results of most phase I trials with first generation CARs are clinically disappointing they proved safety and feasibility and are a first example of investigator-initiated bench-to-bedside translational science. Major problems of first-generation CARs are their lack of expansion and persistence *in vivo*. This may result from eliciting an

immune response directed against the CAR and the subsequent elimination of re-directed T cells (Lamers et al., 2006) and/or sub-optimal culture conditions of T cells prior to infusion. Furthermore, it is becoming more apparent that a major factor contributing to poor persistence and function is the inability of the CAR to provide co-stimulatory events and fully activate the T cell without rendering it anergic.

To compensate the lack of co-stimulation two approaches are currently explored. The first approach comprises the incorporation of various co-stimulatory domains in the CAR construct. Binding of these CARs to their specific antigen results in T cell activation, proliferation and IL-2 secretion without the need of cross-presentation by DCs (Maher et al., 2002; Vera et al., 2006). The second approach for providing co-stimulation targets antigen-specific CTLs which recognize through their native $\alpha\beta$ TCR an antigen present on APCs. Thereby, T cells are getting re-stimulated in a physiological way and the CAR functions to re-direct those (Pule et al., 2008).

1.6.6 Phase I clinical studies employing second and third generation CARs

Currently, several clinical studies using second and third generation CARs are in progress. The group of Dr. Carl June demonstrated objective clinical responses in their recent trial (Porter et al., 2011) utilizing a second generation CAR construct against CD19 in patients with chronic lymphoid leukemia. Re-infusion of these CAR^+ T cells led to complete remission of chronic lymphoid leukemia in 2/3 patients and one partial remission (Kalos et al., 2011). Although, not many clinical responses have been reported up to now, two severe adverse events have occurred resulting in the patients´ death. One report by Morgan et al. stated the administration of a third-generation CAR directed against ERBB2. This resulted in immediate pulmonary toxicity and post mortem analyses revealed spiking serum cytokine levels. Most likely, low levels of ERBB2 expression on normal lung epithelial cells led to a cytokine storm and the rapid decline of the patient (Morgan et al., 2010b). The second incident was reported after administration of a second-generation CAR re-directed against CD19 treating refractory chronic lymphoid leukemia patients. The patient became hypotensive and developed acute renal failure after adoptive T cell transfer. All signs were consistent with the clinical picture of acute sepsis and could not be traced back to the occurrence of tumor lysis syndrome. Therefore, the clinical trial protocol was modified and consequently fewer CAR positive T cells are infused into patients (Brentjens et al., 2010).

1.6.7 Strategies to limit unforeseen toxicity events

As exemplified in these two unforeseen adverse events eminently attention needs to be directed towards toxicity effects of CAR re-directed T cells related to their distribution in normal tissue after adoptive transfer. Intravenous application of re-directed T cells shows antigen-independent pooling in the lungs and liver (Morgan et al., 2010b; Parente-Pereira et al., 2011). Furthermore, unwanted on-target effects describe the recognition of antigen expressed on non-malignant cells which result in antigen-specific activation of CAR re-directed T cells outside the tumor tissue. This may result in (i) immediate cytotoxicity and /or (ii) late or sustained toxicity leading to the long term depletion of cells (Ertl et al., 2011). Immediate cytotoxicity may occur directly after infusion of re-directed T cells when massive activation can lead to the patient's death. Special precautions need to be taken if CARs are directed against untested or endogenous antigens as high avidity receptors recognize their antigen and activate T cells at antigen levels which cannot be detected by conventional methods. Limiting such toxicity is achieved by employing dose-escalation schemes, splitting the T cell dose over several days (Brentjens et al., 2010) or using first generation CARs. The additional insertion of conditional suicide genes has limited potential for preventing acute toxicities as CAR T cells may act within minutes and symptoms only occur after severe damage has already happened (Morgan et al., 2010b). The other risk of long term depletion of cells necessary for normal function is exemplified for B cell malignancies which are treated with CAR T cells directed against CD19 or CD20. If CAR T cells would be persistent and functional a continuous depletion of B cells would be the result. If the chosen antigen is expressed in healthy tissue, like VEGF receptor 2 (VEGFR2) which is needed during angiogenesis, vasculogenesis, and wound healing etc. (Shibuya et al., 2006) the resulting medical conditions could be treated with drugs or alternatively, a suicide gene could be inserted in CAR T cells.

1.6.8 Adoptive T cell therapy for multiple myeloma

Multiple Myeloma (MM) is the second most common blood borne malignant disease and is characterized by neoplastic transformed plasma cells in the bone marrow (Palumbo and Anderson, 2011). Though extensive treatment regimens are available, MM is still considered an incurable disease. The immune system seems to be a critical factor for disease control in multiple myeloma since immune-modulatory drugs and allogeneic stem cell transplantation can achieve long lasting remissions in a subgroup of patients (Giaccone et al., 2011; Kyle and Rajkumar 2004). The curative effect of allogeneic stem cell transfer is, in part, an immunological effect mediated by T cells from the donor defined as graft versus myeloma effect (Bleakley and Riddell, 2004). Antigens expressed on

myeloma cells allowing graft versus myeloma effects are mainly minor histocompatibility antigens (Hambach and Goulmey, 2004). However, the recognition of minor histocompatibility antigens is associated with graft-versus host disease (GvHD), one of the most serious complications in allogeneic stem cell transfer. Therefore, the identification of new myeloma specific markers unrelated to histocompatibility antigens is of great interest.

NY-ESO-1 is a promising target for immunotherapy, because NY-ESO-1 expression is found in malignant plasma cells with expression levels of 30 % at first diagnosis and up to 60 % at relapse (van Rhee et al., 2005). At the National Institutes of Health (Bethesda, USA) are currently two phase I/II trials listed which recruit patients with advanced myeloma. In one trial, the investigators will infuse autologous genetically modified T cell to express a high affinity NY-ESO-1 TCR in HLA-A*02 positive patients and a high affinity Mage-A3 TCR in HLA-A*01 patients. Furthermore, one phase I clinical trial for multiple myeloma patients is conducted using autologous T cells engrafted with a CAR targeting the kappa light chain of immunoglobulins. These trial will also compare the functionality of first and second generation CAR grafted T cells.

1.7 Aims of the thesis

Adoptive T cell therapy with re-directed CAR^+ T cells seems to be a highly promising approach to treat cancer. A widely expressed TAA is NY-ESO-1. Due to the intracellular location of the protein, it first needs to be degraded to peptide fragments, loaded onto HLA class I molecules and transported to the cell surface to be presented to $CD8^+$ T cells. Multiple naturally occurring regulatory mechanisms of the immune system as well as the immunosuppressive tumor environment may lead to a deprivation of $CD8^+$ T cells specific for NY-ESO-1 proteins. In prior studies, our group selected by phage display a Fab fragment which recognizes the NY-ESO-$1_{157-165}$ peptide in the HLA-A*02:01 context. The corresponding scFv was used to construct a CAR to re-direct $CD8^+$ T cells. The overall goal of the thesis project is to proof antigen-specific functionality of NY-ESO-$1_{157-165}$/HLA-A*02:01 specific re-directed T cells which ultimately should lead to the initiation of a phase I clinical trial for HLA-A*02:01 positive patients with NY-ESO-1 positive multiple myeloma. To demonstrate feasibility and functionality the following aims were specified:

1. to generate first and second generation CAR constructs which recognize in a TCR manner the NY-ESO-$1_{157-165}$/HLA-A*02:01 complex
2. to investigate the functional potential of the generated re-directed T cells *in vitro*
3. to phenotypically analyze subpopulations of the re-directed T cells
4. to determine functional differences of the found subpopulations *in vitro*

5. to explore the *in vivo* functionality of re-directed T cells

2 Material and Methods

2.1 Equipment

Agarose Gel Documentation BioDoc-It Imaging System	UVP, Upland, USA
Autoclave V-100	Systec, Hühnenberg, Switzerland
Cell counter CASY Cell Counter Model TT	Roche Innovatis, Bielefeld, Germany
Centrifuges: Centrifuge 5804R Centrifuge 5810R Centrifuge 5415D	Eppendorf, Hamburg, Germany
Flow Cytometers and Sorters: FACScan FACSCalibur FACSCanto II LSR II FACSAria III	BD Biosciences, San Jose, USA
Magnetic activated cell sorting MACS multistand & quadro MACS	Miltenyi Biotec, Bergisch Gladbach, Germany
NanoDrop 1000 Spectrometer	ThermoScientific Wilmington, USA
PCR cycler T300 Thermocycler	Biometra, Göttingen, Germany
Plate reader Wallac Victor^2 1420 Multilabel Counter	PerkinElmer, Waltham, USA
Water purification Milli-Q Gradient System	Millipore, Bedford, USA

2.2 Plasmids and bacteria

2.2.1 Plasmids

- **pCOLT-GalV (#392)**

  Kind gift of Prof. Abken, Cologne, Germany;

  First described by Weijtens et al., 1998;

  Retroviral helper plasmid coding for GALV env protein

- **pHIT60 (#393)**

  Kind gift of Prof. Abken, Cologne, Germany;

  First described by Weijtens et al., 1998;

  Retroviral helper plasmid coding for MLV gag and MLV pol protein

- **BW431/26-CD28/CD3ζ (#607)**

  Kind gift of Prof. Abken, Cologne, Germany;

  First described by Hombach et al., 2001;

  Plasmid coding for a CEA recognizing chimeric antigen receptor expressing CD28 and CD3ζ

  Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, a scFv recognizing carcinoembryonic antigen (CEA), a human CH_2CH_3 IgG domain and a CD28/CD3ζ domain.

- **3M4E5-CD3ζ (#1044wt)**

  Generated in our lab;

  Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, the wt 3M4E5 scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a human CH_2CH_3 IgG domain and a CD3ζ domain.

- **3M4E5-CD28/CD3ζ (#1046wt)**

  Generated in our lab;

  First described by Stewart-Jones et al., 2009

  Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, the wt 3M4E5 scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a human CH_2CH_3 IgG domain and a CD28/CD3ζ domain.

- **T1-CD3ζ (#1044t1)**

  Generated in our lab;

  Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, the T1 scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a human CH_2CH_3 IgG domain and a CD3ζ domain.

- **T1-CD28/CD3ζ (#1046t1)**

  Generated in our lab;

  First described by Stewart-Jones et al., 2009

  Expression cassette codes for a fusion protein consisting of a Lκ-leader sequence, the T1 scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a human CH_2CH_3 IgG domain and a CD28/CD3ζ domain.

2.2.2 Bacterial strain

One shot TOP10 Chemically Competent *E.coli*	Invitrogen, Karlsbad, USA

2.3 Media for bacterial growth

- **LB medium**

  10 g Bacto Tryptone (BD, Sparks, USA)
  5 g Yeast extract (Sigma-Aldrich, St.Louis, USA)
  5 g NaCl
  1 l dH_2O
  Medium was heat sterilized

- **LB-Amp**

  LB medium with 100 μg/ml ampicillin (Sigma-Aldrich, St. Louis, USA) added after heat sterilization

- **LB-Amp plates**

  LB-Amp with 1.5 % (w/v) Difco Agar (BD, Sparks, USA) added before sterilization

2.4 Enzymes

Phusion High-Fidelity DNA Polymerase	Finnzymes, Espoo, Finland
Restriction enzyme NcoI	New England BioLabs, Ipswich, USA
Restriction enzyme BamH1	New England BioLabs, Ipswich, USA
Streptavidin-Horse Radish Peroxidase	BD Biosciences, San Diego, USA

2.5 Primer

Primer designation	Primer sequence (5'-3')
f1-Part2Lkappa-3M	CTA ATC AGT GCC TCA GTC ATA ATG TCT AGA ATG GCG GAG GTG CAG CTG
f2-NcoI-Part1Lka	CGT ACC ATG GAT TTT CAG TGT CAG ATT TTC AGC TTC ATG CTA ATC AGT GCC TCA GTC ATA ATG TC
r-BamH1-3M4E5	GAT AGG ATC CAC TGT GGG GTT GG
f-VH-peptide linker	ATG GAT GTG TGG GGC CAG G
f-CD3	AGG ACA CCT ACG ACG CCC TT

Table 2-1: Primer designations and primer sequences

2.6 Buffers, DNA dyes and ladders

Chemical reagents listed here were purchased from the following manufacturers: Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, USA), Kantonsapotheke (Zürich, Switzerland), Roth (Karlsruhe, Germany); Fluka (Buchs, Switzerland). DNA ladders were purchased from New England BioLabs (Ipswich, USA).

2.6.1 Buffers

- **TAE**

2 mM	TRIS Ultra
0.25 mM	Acetic acid
0.5 mM	EDTA

- **PBS**

150 mM	NaCl
10 mM	Na_2HPO_4
1.5 mM	KH_2PO4

- **0.05 % PBST**

99.95 % (v/v)	PBS
0.05 % (v/v)	Tween20

- **Flow cytometry buffer**

2 % (v/v)	FBS, heat inactivated
0.01 % (w/v)	Sodium azide
5 mM	EDTA
1 l	PBS

- **Blocking solution**

10 % (v/v)	FBS, heat inactivated
90 % (v/v)	PBS

- **Triton X buffer**

150 mM	NaCl
50 mM	Tris-HCl
10 mM	$MgCl_2$
1 % (w/v)	TritonX

2.6.2 DNA dyes and ladders

- **5x DNA loading dye**

50 % (v/v)	TAE
50 % (v/v)	Glycerol
0.4 % (w/v)	Orange G

- **DNA ladder**

20 % (v/v)	100 bp or 1 kb DNA ladder (New England BioLabs, Ipswich, USA)
40 % (v/v)	5x DNA loading dye
40 % (v/v)	dH_20

2.7 Antibodies, Fab molecules, tetramers, conjugates, and cytokines

2.7.1 Antibodies

Anti-human HLA-A,B,C-FITC	Biolegend, San Diego, USA
Mouse IgG2a, κ – FITC Isotype control	Biolegend, San Diego, USA
Anti-human CD8-FITC	eBioscience, San Diego, USA
Anti-human CD3-PE-Cy7	BD Biosciences, San Jose, USA
Anti-human IgG-PE	Southern Biotech, Birmingham, USA
Anti-human CCR7-FITC	eBioscience, San Diego, USA
Anti-human CD45RA-PerCp-Cy5.5	eBioscience, San Diego, USA
Anti-human CD57-Biotin	BD Biosciences, San Jose, USA
Anti-human CD28-APC	BD Biosciences, San Jose, USA
Anti-human CD27 APC-H7	BD Biosciences, San Jose, USA
Anti-human IFNγ-PE-Cy7	BD Biosciences, San Jose, USA
Anti-human IL-2-PerCp-eFlour710	eBioscience, San Diego, USA
Anti-human CD8 microbeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-human IgE antibody	Biolegend, San Diego, USA
Biotin-AffiniPure F(ab')2 fragment goat anti-human IgG (H+L)	Jackson Immuno Research, Suffolk, UK
Human OKT3	eBioscience, San Diego, USA

Human CD28 (co-stimulatory)	eBioscience, San Diego, USA

2.7.2 Tetramer

PE conjugated HLA-A*02:01/NY-ESO-$1_{157-165}$ tetramer	LICR, Lausanne, Switzerland

2.7.3 Conjugate

Streptavidin-PE-Cy7	eBioscience, San Diego, USA

2.7.4 Fab molecules

AI anti-idiotype Fab A4	in house, Zurich, Switzerland
Control Fab (B1)	in house, Zurich, Switzerland

2.7.5 Cytokines

Recombinant human IL-2	ImmunoTools, Friesoythe, Germany
Recombinant human IL-15	ImmunoTools, Friesoythe, Germany

2.8 Kits

BD Cytofix/Cytoperm Kit	BD Biosciences, San Diego,USA
BD OptEIA Set Human IFNγ	BD Biosciences, San Diego,USA
BD OptEIA Set Human IL-2	BD Biosciences, San Diego,USA
QIAGEN Plasmid Maxi Kit	QIAGEN, Hilden, Germany
QIAprep Spin Miniprep Kit	QIAGEN, Hilden, Germany
QIAquick Gel Extraction	QIAGEN, Hilden, Germany
Rapid DNA Ligation Kit	Roche Diagnostics, Mannheim, Germany
REDTaq Ready Mix	Sigma-Aldrich, St. Louis, USA
DELFIA Eu labelling Kit	Perkin Elmer, Waltham, USA
MycoAlert Mycoplasma Detection Kit	Lonza Cologne GmbH, Cologne, Germany

2.9 Cell culture media, reagents and additives

RPMI 1640 media with Glutamax	Gibco, Carlsbad, USA
Hygromycin B	Gibco, Carlsbad, USA
Penicillin/Streptomycin	Gibco, Carlsbad, USA
Fetal Bovine Serum (FBS) Heat inactivation: 56 °C for 30 min	Gibco, Carlsbad, USA
0.05 % Trypsin-EDTA	Gibco, Carlsbad, USA
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany

2.10 Cell lines and primary cells

- **293T**
  Kind gift of Prof. Abken, Cologne, Germany
  Derived from 293 cells, carries the SV40 large T antigen
- **U266**
  Kind gift of Prof. Keilholz, Charité, Berlin
  Human multiple myeloma cell line, secrets hIgE molecules
- **T2-1B**
  Held et al., 2004
  Minigene-transfected T2 cell line expressing NY-ESO-$1_{157\text{-}165}$/HLA-A*02:01-complexes
- **T2-1A**
  Held et al., 2004
  Minigene-transfected T2 cell line expressing NY-ESO-$1_{157\text{-}167}$/HLA-A*02:01-complexes

2.11 Mice

Five to eight weeks old female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice, commonly known as NOD scid gamma (NSG), were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and maintained under specific pathogen-free conditions on Institutional Animal Care and Use Committees (IACUC) protocols.

2.12 Software

Cell Quest Pro 4.0.2	BD Biosciences, San Jose, USA
FACSDiva	BD Biosciences, San Jose, USA
FlowJo 7.5	Treestar, Ashland, USA

GraphPad Prism V 5.01 for Windows	Graph Pad Software, San Diego, USA
Microsoft Office Excel 2003	Microsoft, Redmond, USA

2.13 Bacterial culture methods

2.13.1 Growth of bacteria in suspension

For bacterial expansion, LB-Amp media was inoculated with 15 µl of bacteria from a respective glycerol stock and incubated over night at 37 °C and 250 rpm.

2.13.2 Growth of bacteria on agar plates

Freshly transformed Top10 bacteria in SOC media were plated on LB-Amp agar plates over night at 37 °C.

2.13.3 Glycerol stocks of bacteria

Bacterial suspension was diluted with glycerol (50 % (v/v), Sigma-Aldrich, St.Louis, USA) and after thorough mixing stored at -80 °C.

2.13.4 Transformation of competent *E.coli* cells

After ligation of DNA fragments, the mixture was added to one vial of TOP10 competent *E.coli* cells and incubated for 30 min on ice. Cells were heat-shocked for 45 s at 42 °C and directly afterwards placed on ice for 1 min. 250 µl SOC media were added followed by a 1 hour incubation period at 37 °C and 250 rpm.

2.14 Molecular biology techniques

2.14.1 Purification of DNA

For purification of DNA several kits were used according to the manufacturer's protocol.

DNA fragments which have been separated by gel electrophoresis were recovered using QIAquick Gel Extraction kit. Small and large amounts of plasmid DNA were purified from *E.coli* bacteria using the QIAprep Spin Miniprep kit or the QIAGEN Plasmid Maxi kit, respectively.

2.14.2 Determination of DNA concentration

Using the NanoDrop DNA concentration was determined by measuring the absorbance at 260 nm and at 280 nm to account for impurities. DNA with an A260 nm/A280 nm of 1.8-2 was considered as pure.

2.14.3 Agarose gel electrophoresis

1 % (w/v) agarose gels prepared with TAE buffer and 10 μg/ml ethidium bromide (EtBr) were used for analytical or preparative separation of DNA fragments. The DNA was mixed with a 5x DNA loading dye. 100 bp and 1 kb DNA ladders were applied to individual gel pockets as size standards. A voltage of 100-120 V was applied to the gels. Separation of DNA fragments was monitored under UV light.

2.14.4 DNA amplification by polymerase chain reaction (PCR)

The principle of PCR is thermal cycling, comprising of repeated cycles of DNA melting and the subsequent enzymatic amplification of DNA pieces. Thereby are several magnitudes of copies of a particular DNA sequence generated.

The reaction was carried out in a temperature controlled T300 thermocycler.

Reaction conditions for DNA amplification were as follows:

Template DNA	10-100 ng
Forward Primer	20 pmol
Reverse Primer	20 pmol
dNTPs	200 μM
Phusion polymerase	1 U
5x GC buffer	10 μl
dH_20	volume was adjusted to 50 μl

Following cycling conditions were applied:

Initial denaturation	96 °C	6 min
Denaturation	95 °C	45 s
Annealing	55 °C	45 s
Elongation	72 °C	1 min, back to step 2, 30 cycles
Final elongation	72 °C	10 min

2.14.5 Digestion of DNA with restriction endonucleases

Digestion of vector and PCR products was conducted in two steps as follows:

Step 1:	Template	1 μg
	NcoI	1 U
	NEB buffer #4	5 μl
	dH_2O	volume was adjusted to 50 μl
	Incubation at 37 °C for 30 min	
Step 2:	1 U BamHI was added to the reaction	
	Incubation at 37 °C for 30 min	

2.14.6 Ligation of DNA

DNA fragments were ligated using the Rapid DNA Ligation kit according to the manufacturer's protocol. The insert: vector ratio was 3:1.

2.14.7 Colony PCR

To verify correct insertion of the DNA fragment of interest into the plasmid a colony PCR reaction was carried out with the REDTaq ReadyMix according to the manufacturer's protocol and using the primer pairs f2-NcoI-Part1Lka and r-BamHI-2M4E5. Cycling conditions for amplification of DNA were applied. The reaction was analyzed via agarose gel electrophoresis.

2.14.8 Sequencing

Successful cloning of all plasmids was confirmed by sequencing the flanking regions of the insertion site (primers: f2-NcoI-Part1Lka, f-VH-peptide linker, and f-CD3). Sequencing was performed by Microsynth AG (Balgach, Switzerland).

2.14.9 Cloning strategy of high affinity chimeric antigen receptors

To construct retroviral expression vectors plasmids were used derived from the retroviral expression vector pBullet. pBullet codes for a SV40 ori, an ampicillin resistance and a CMV promoter controlling the expression cassette. It is derived from pStitch by deleting two NcoI and one XhoI restriction sites (Weijtens et al., 1998).

In preliminary studies our group isolated two Fab molecules recognizing the NY-ESO-$1_{157-165}$ peptide in the context of HLA-A*02:01 molecules. They differ in their affinity to bind their specific antigen: the wild type (3M4E5) variant possesses a K_D value around 60 nM and the type1 (T1) variant around 2 nM. Based on the sequences of these Fab molecules single chain Fv (scFv) fragments were generated (Stewart-Jones et al., 2009).

3M4E5-CD28/CD3ζ and 3M4E5-CD3ζ each cloned into one individual pBullet vector were used as starting vectors (Stewart-Jones et al., 2009). Their expression cassette codes for a

fusion protein consisting of a Lκ-leader sequence, a scFv recognizing the HLA-A*02:01/NY-ESO-$1_{157-165}$ complex, a CH_2CH_3 IgG domain and a CD28/CD3ζ domain or a CD3ζ domain, only. To exchange the 3M4E5 scFv with the T1 scFv, the Lκ leader sequence and the appropriate restriction sites were added to the T1 scFv in two PCR steps using in a first step the primer pair f1-part2Lkappa-3M and r-BamHI-3M4E5 and in a second step pair f2-NcoI-Part1Lka and r-BamHI-3M4E5. PCR products were purified via gel electrophoresis and subsequent gel extraction. 3M4E5-CD28/CD3ζ and 3M4E5-CD3ζ vectors and the end PCR product of Lκ-T1 scFv were digested with NcoI and BamHI and ligated. Successful insertion of the Lκ-T1 scFv was analyzed by colony PCR and verified by sequencing.

2.15 Cell culture techniques and immunobiological methods

2.15.1 Cultivation of cell lines

Stable-transfected HLA-A*02:01-positive T2 cell lines 1A and 1B expressing HLA-A*02:01 restricted NY-ESO-1 peptides 157-167 (T2-1A) and 157-165 (T2-1B), respectively were cultivated in R10 media [RPMI1640 GlutaMax supplemented with 10 % fetal bovine serum (FBS) (v/v), 50 U/ml penicillin and 50 μg/ml streptomycin] supplemented with 2.5 μg/ml hygromycin B. 293T and U266 cells were cultivated in standard R10 media only. All cells were cultured at 37 °C and 5 % CO_2. Adherent cell lines were detached from cell culture flasks using trypsin-EDTA. Cells were frozen down using freezing media [90 % (v/v) FBS, 10 % (v/v) DMSO].

2.15.2 Mycoplasma test

Every three months, each cell line was tested for mycoplasma using MycoAlert Mycoplasma Detection Kit (Lonza Cologne GmbH, Cologne, Germany) according to the manufacturer's protocol.

2.15.3 Determination of cell number and viability

Cell number, size distribution and viability of cell lines and primary human cells were determined by CASY Cell Counter + Analyzer System Model TT. The method is based on electrical current exclusion which measures the volume and viability of the cell in a dye-free setting.

2.15.4 Retroviral transduction of peripheral blood $CD8^+$ T cells

Peripheral blood mononuclear cells were obtained from healthy donors by Ficoll PaqueTM Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient centrifugation. CD8 T cell subsets were isolated using MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Positive selection with anti-human CD8 microbeads typically resulted in a ≥ 95% pure $CD8^+$ population. Purified $CD8^+$ T cells were cultivated for 48 h in R10 media in the presence of 400 IU/ml human recombinant IL-2, anti-human OKT3 mAb and anti-human CD28 mAb (each at 100 ng/ml).

For production of infectious gibbon-ape leukemia virus pseudotyped retroviruses, 293T cells were seeded at $2.5*10^5$ cells/ well (6-well plate) and co-transfected with the retroviral helper plasmids pHIT60 and pCOLT (each 0.75 µg DNA) together with the retroviral expression vector DNA (1.5 µg DNA) using 12 µl Fugene transfection reagent per well (Roche Diagnostics GmBH, Mannheim, Germany, according to the manufacturer's protocol). If CAR transduced $CD8^+$ T cells were used for *in vivo* studies, $7.5*10^5$ 293T cells/ well were plated and irradiated with a single dose of 3000 Rad after 24 h of co-transfection.

Activated $CD8^+$ T cells were infected for 96 hours in the presence of 100 IU/ml IL-2 by co-cultivation with 293T cells which are transiently producing high titers of infectious retrovirus. $CD8^+$ T cells were harvested and monitored for expression of CAR using flow cytometry.

2.15.5 Immunoflourescence staining and analyses

Cells were washed in flow cytometry buffer and surface stained with directly labeled or biotinylated antibodies (20 min at 4°C), or with peptide-MHC class I tetramer complexes (30 min at RT). Thereafter, cells were fixed using 500 µl of FACSLyse buffer (BD Biosciences, San Diego, USA) (10 min at RT). Cells were then washed, resuspended in flow cytometry buffer and stained with tandem conjugates (20 min at RT). After washing, cells were resuspended in flow cytometry buffer and analyzed by flow cytometry.

For intracellular cytokine staining, cells were stimulated with 2 µg/ml AI anti-idiotype Fab A4 or control Fab for 12 h in the presence of Golgi Plug (BD Biosciences, San Diego, USA). Cell surface staining was performed as described above, followed by fixation and permeabilization using 250 µl of Cytofix/Cytoperm solution (20 min at 4 °C). After washing with 1x Perm/Wash buffer (BD Biosciences, San Diego, USA), cells were stained with directly labeled antibodies against IL-2 and IFNγ (30 min at 4 °C). After another round of washing, flow cytometry analyses was performed using a LSR II, FACSCanto II, FACSCalibur or FACScan flow cytometer. All data were analyzed using FlowJo software. At least 150,000 cells were collected for 6- and 4-colour flow cytometry analyses.

2.15.6 Cell Sort

To isolate $CCR7^+$ and $CCR7^-$ CAR-grafted T cell subsets, $CD8^+$ T cells were stained with hIgG and CCR7 antibody and sorted to > 90 % purity using FACSAria III. Cells were either used for *in vivo* experiments or plated in a pre-coated 96-well flat bottom plate (2 µg/ml of AI anti-idiotype Fab and control Fab overnight, 4 °C) at a density ranging from $1.6*10^4$/well to $3.7*10^5$/well depending on the sort efficiency and incubated in standard media supplemented with 50 ng/ml IL-15 for a total of 10 days at 37 °C. After 24 h incubation, supernatants were taken for cytokine monitoring and cells were stained for hIgG and CCR7 expression and analyzed via flow cytometry after 10 days of incubation.

2.15.7 Cytokine and hIgE secretion assays

Cytokine production was assessed by performing sandwich ELISA assays. Therefore, supernatants of co-cultivated effector and target cells were collected after 24 hours of incubation. IFNγ and IL-2 levels were detected using BD OptEIA set human IFNγ and BD OptEIA set human IL-2 kits, respectively, according to the manufacturer's instruction.

To assess tumor burden, mouse blood was taken from the tail vein, centrifuged for 20 min at 1500 rpm, RT and the sera was collected. Subsequently, sera levels of hIgE proteins were measured. For that purpose, 96-well maxisorp microtiter plates (NUNC, Rochester, USA) were coated with 1 ug/ml purified anti-human IgE antibody diluted in carbonate buffer (1 h at 37 °C). Plates were washed three times in 0.05% Tween20/PBS and then blocked with 10 % FBS/PBS (1h at RT). After three washing steps, samples and standard (human IgE protein, Abcam, Cambridge, USA) were added to the plate and incubated (2 h at RT). The plates were washed five times and then incubated (1 h at RT) with 1 ug/ml Biotin- AffiniPure F(ab')2 fragment goat anti-human IgG (H+L) diluted in 10 % FBS/PBS. After five washing steps, 1 ug/ml Streptavidin-Horse Radish Peroxidase was added (1 h at RT). Plates were washed five times and ELISAs developed with SureBlue (KPL, Gaithersburg, MD), stopped using H_3PO_4 (Sigma-Aldrich, St. Louis, USA) and measured at 450 nm. The detection limit for human IgE was 0.125 ng/ml.

2.15.8 Europium release assay

Specific cytotoxicity of CAR grafted T cells was analyzed by a europium release assay according to the manufacturer's protocol (Perkin Elmer, Waltham, USA) with minor modifications. Briefly, target cells (1A, 1B and U266) were labeled with 2,2':6',2"-terpyridine-6,6"- dicarboxylic acid acetoxymethylester (BATDA) (2 h at 37 °C). After 5 washing steps, target cells were seeded at a density of 10^4 cells per 96-well and co-cultured with effector cells at different effector: target (E/T) ratios (2 h at 37 °C). Supernatant was harvested and

incubated with DELFIA Europium solution (15 min at RT) under constant vibration. The ligand released from dead cells forms together with europium a highly stable fluorescent chelate (EuTDA) which was measured in a time-resolved Victor^2 fluorometer. Maximal EuTDA formation was determined as the mean of three wells of target cells lysed with TritonX buffer. Background and spontaneous release were determined from triplicate wells containing R10 media only or target cells alone, respectively.

The specific cytolysis of target cells (%) was calculated by the equation:

$$specific\ cytolysis\ of\ target\ cell(\%) = 100 * \frac{(experimental\ release\ (counts) - spontaneous\ release\ (counts))}{(maximum\ release\ (counts) - spontaneous\ release\ (counts))}$$

2.16 Xenograft model

To investigate the *in vivo* potential of re-directed T cells, an immuno-compromised mouse model was employed, so called NSG mice (strain name: NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ). This mutant mouse strain combines the NOD.SCID background with an IL-2 receptor gamma chain deficiency. As a consequence, these mice lack mature B cells, T cells, and functional NK cells. Furthermore, they are deficient in cytokine signaling which leads to a better engraftment of hematopoietic stem cells and peripheral blood mononuclear cells (PBMCs). Following irradiation of mice, intravenous injection of the human multiple myeloma cell line U266 resembles the pathogenesis of the disease (Miyakawa et al., 2004). Additionally, U266 cells are HLA-A*02:01$^+$ and express endogenously NY-ESO-1 making them an excellent target for NY-ESO-1$_{157-165}$/HLA-A*02:01 CAR expressing T cells.

2.16.1 *In vivo* tumor growth

Mice were irradiated on a single fraction of 2.4 Gy followed by subsequent injection of either 2*10^7 U266 cells intravenously (i.v.), 10^7 U266 cells i.v., 6*10^6 U266 cells i.v. or 2*10^7 U266 cells intraperitoneally (i.p.). *In vitro*, 10^6 U266 cells secrete about 300 ng/ml of human hIgE in 24 hours. To monitor growth of malignant plasma cells, blood samples were taken after inoculation. Sera were collected and hIgE production was analyzed by ELISA (material and methods section 3.15.6) as indicated by figure 2-1.

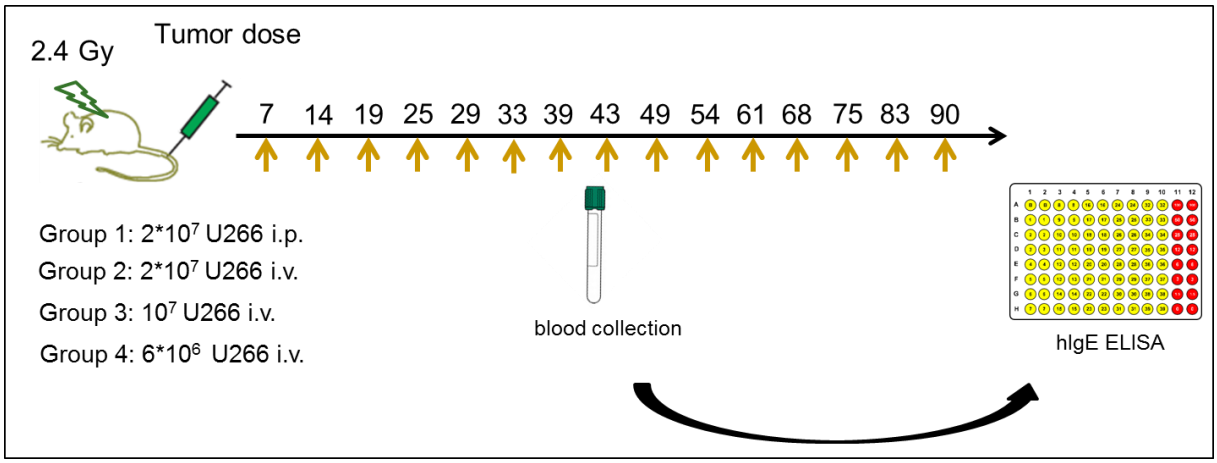

Figure 2-1: Establishment of *in vivo* growth of U266 cells.

As indicated, four different doses/ injection routes of U266 cells were established into sublethal irradiated NSG mice. The yellow arrows indicate the days of blood draw after U266 injection. Sera were isolated from blood and were used to measure hIgE levels as surrogate marker for tumor growth via ELISA.

2.16.2 Analyses of U266 growth in the bone marrow, spleen, and blood

To confirm U266 growth in the bone marrow of mice, tibia was resected and subsequently decalcified and H&E stained (Pathology Department, Providence Portland Medical Center, Portland, OR, USA).

Furthermore, bone marrow of tibia and femur, spine as well as blood samples and splenocytes were obtained to analyze U266 tumor growth. Spleen tissue was pierced and splenocytes were teased out both using curved watchmaker forceps. Splenocytes and spleen tissue were filtered through a 70 µm sieve. Afterwards, a centrifugation step was carried out to spin down the cells at 1500 rpm for 5 min, RT. The supernatant was discarded and the cells were resuspended in 1 ml ACK buffer for 3 min at RT to lyse erythrocytes. Tibia and femur from mice were excised using scissors and by breaking apart at joints the intact bone was freed from flesh. The bones were cut on both ends to open up the bone. R10 media was aspirated into a syringe, a 27G needle was attached on top. The needle was inserted into the opened bones and the marrow was flushed out (also for spine). The solution was pipetted up and down to break aggregates. The cell solution was filtered through a 70 µm sieve, pelleted and resuspended in 1 ml ACK buffer for 3 min at RT. Blood samples were collected from the tail vein and erythrocytes were lysed using ACK. All samples were stained with monoclonal antibodies as described in material and methods section 3.15.4.

2.16.3 Treatment protocols of U266 tumors

Five days after 10^7 U266 tumor cell i.v. injection, different doses of re-directed $CD8^+$ T cells were administered i.v. followed by six bi-daily injections of 90 000 IU IL-2. The treatment protocol is displayed in figure 2-2. The number of BW431/26-CD28/CD3ζ re-directed T cells

administered to the control groups was adjusted according to the transduction efficiency. 50 % of T cells expressed CARs, irrespective of the individual constructs.

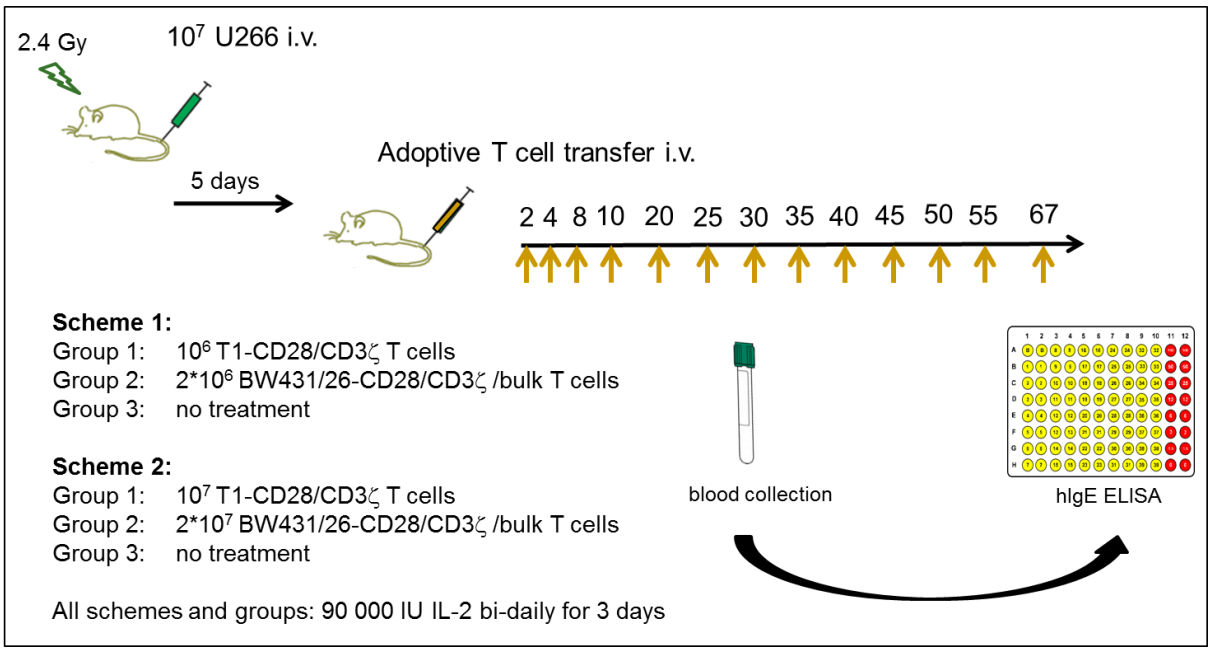

Figure 2-2: Treatment protocols of U266 tumors.

Treatment started after 5 days of i.v. injection of 10^7 U266 cells into sublethal irradiated NSG mice. In a first setting, mice were i.v. injected with 10^6 T1-CD28/CD3ζ T cells (transduction efficiency 50 %) and with the same amount of BW431/26-CD28/CD3ζ/ bulk T cells as specificity control. Group 3 served as tumor growth control. In a second setting, tumor bearing mice were treated with 10^7 T1-CD28/CD3ζ T cells and their number equivalent of BW431/26-CD28/CD3ζ/ bulk T cells. As before, group 3 did not receive any treatment.

2.17 Statistical analyses

Data were analyzed with Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Student's unpaired or paired t-tests were performed between two groups of interest. Furthermore, Mann-Whitney U-test was employed to determine significances between different groups of mice.

3 Results

3.1 Generation of re-directed CD8$^+$ effector T cells expressing CARs with specificity for HLA-A*02:01/NY-ESO-1$_{157-165}$ peptide complex

In previous studies, our group isolated a Fab molecule (3M4E5) recognizing the NY-ESO-1$_{157-165}$ peptide in the context of a HLA-A*02:01 molecule. This Fab fragment was affinity matured to achieve higher peptide binding. Two light chain mutations improved the affinity of the newly generated T1 Fab to 2-4 nM compared to the 3M4E5 Fab which possesses a K_D value around 60 nM (Stewart-Jones et al., 2009). A scFv molecule was constructed based on the sequence of the T1-Fab molecule. This scFv was used as antigen binding domain for a CAR construct. Prof. Abken kindly provided his optimized vector system pBULLET already including either the CH2/3 IgG-CD28-CD3 ζ or the CH2/3 IgG-CD3 ζ domain only. The T1 scFv was extended with an Lκ leader sequence and cloned by appropriate restriction digest and subsequent ligation in the two expression cassettes, thereby generating fully functional chimeric antigen receptors (figure 3-1).

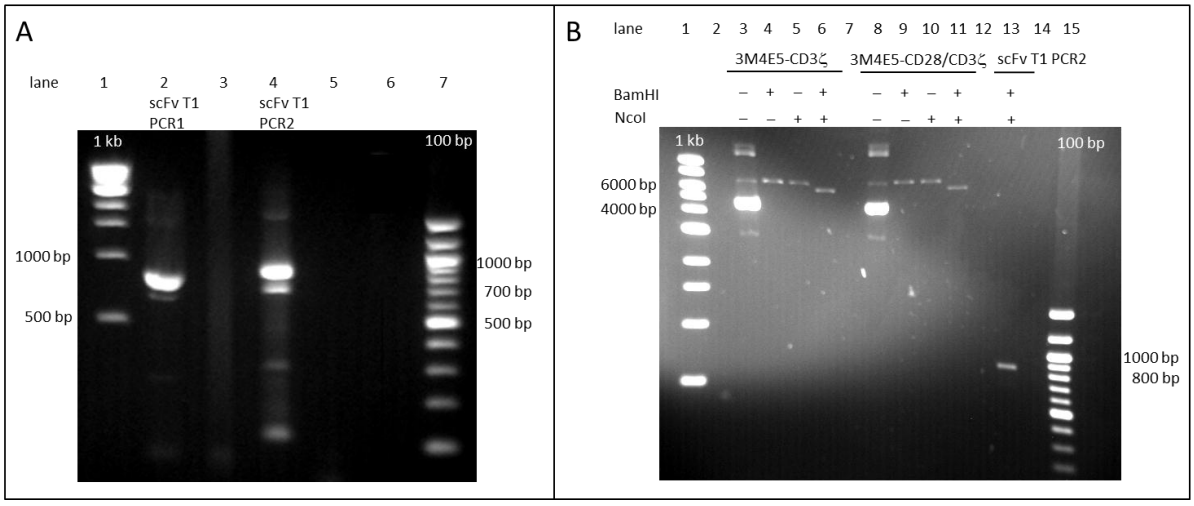

Figure 3-1: **Gel documentation of single PCR and restriction digest steps.**

Panel A shows the prolongation of the T1 scFv via 2 PCR steps. PCR product 1 (PCR1) revealed a size of 803 bp. This was used as template for PCR2 which resulted in a size of 837 bp, comprising the complete sequence of the Lκ leader together with the T1 scFv. Figure B depicts the restriction digest of the parental pBullet vectors (3M4E5-CD3ζ and 3M4E5-CD28/CD3ζ) and of scFv T1 PCR2.

To re-direct T cells, human PBMCs were isolated from buffy coats and positively selected for CD8 expression. Retroviruses were generated employing transient co-transfection of 293 T cells with the respective CAR expression cassette and two helper plasmids, one encoding *gag* and *pol*, the other *env*. Co-cultivation of 293T cells with activated human CD8$^+$ T cells

led to retroviral transduction resulting in the generation of re-directed T cells expressing CARs. These CARs consisted of a scFv molecule recognizing the HLA-A*02:01/ NY-ESO-$1_{157-165}$ peptide complex linked to a human CH2/3 immunoglobulin domain and a CD3ζ signaling domain with (T1-CD28/CD3 ζ) and without (T1-CD3 ζ) a CD28 co-stimulatory domain. Re-directed T cells expressing a CAR recognizing carcinoembryonic antigen (CEA) with a CD28 co-stimulatory domain and a CD3ζ signaling domain (BW431/26-CD28/CD3 ζ, kind gift of Prof. Abken) were used as control (Hombach et al., 2001). The schematic representation of the re-directed T cells is shown in figure 3-2.

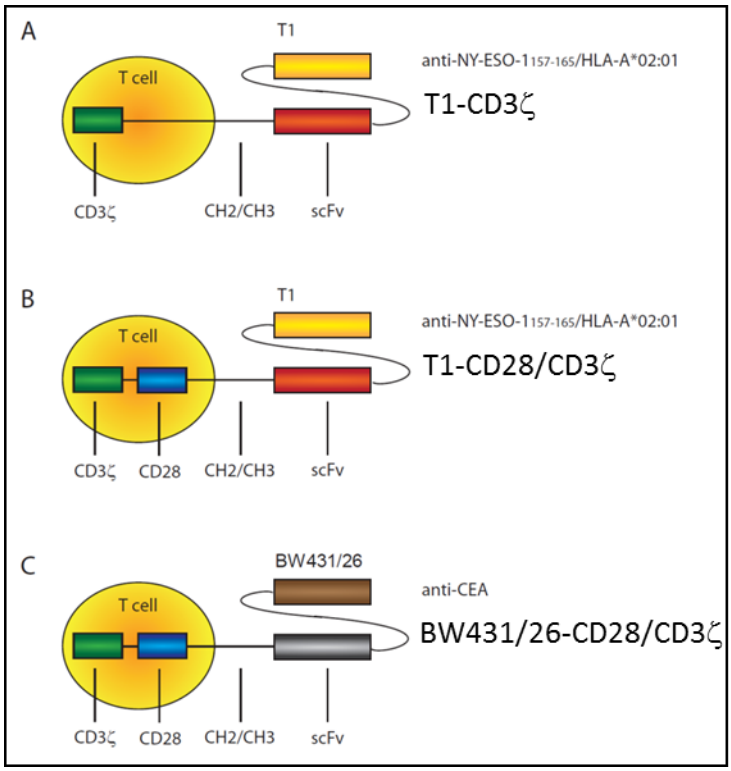

Figure 3-2: Schematic presentation of re-directed T cells including the different CARs.

A: Re-directed T cells recognizing NY-ESO-$1_{157-165}$ peptide in the context of HLA-A*02:01 without CD28 co-stimulatory (T1-CD3ζ) domain; B: Re-directed T cells recognizing NY-ESO-$1_{157-165}$ peptide in the context of HLA-A*02:01 with CD28 co-stimulatory domain (T1-CD28 /CD3ζ); C: Re-directed T cells recognizing CEA with CD28 co-stimulatory domain (BW431/26-CD28/CD3ζ).

Cell surface expression of CARs on retrovirally transduced $CD8^+$ T cells was assessed by detecting the human CH2/3 immunoglobulin domain (hIgG) using flow cytometry (Figure 3-2 A, B, C and D). After transduction, approximately 45% (median 44.42 % for all constructs, range: 11.06% -79.05%) of $CD8^+$ T cells expressed the respective receptor. Antigen recognition of the immune-receptor on the cell surface was measured by HLA-A*02:01/NY-ESO-$1_{157-165}$ peptide tetramer binding (figure 3-3 E, F, G and H). The percentage of T cells expressing the CAR mirrored tetramer staining with no significant difference between constructs T1-CD28/CD3ζ and T1-CD3 ζ. As expected, the tetramer did not bind to the control CAR BW431/26-CD28/CD3 ζ which recognizes CEA.

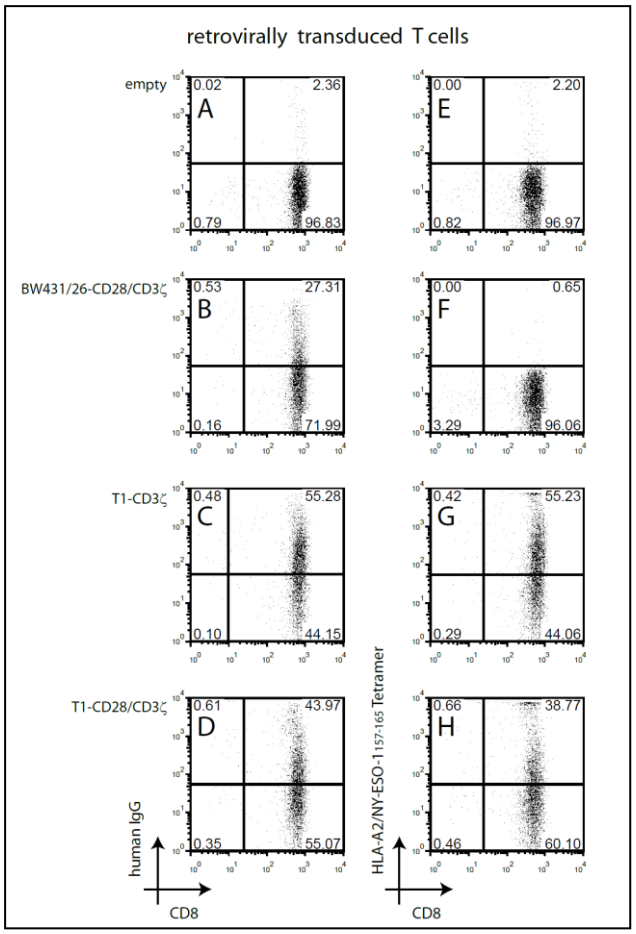

Figure 3-3: Transduction efficacy of CD8$^+$ T cells.

Assessment of transduction efficiency by staining of the CH2/3 immunoglobulin linker domain with anti-hIgG mAb (A-D). Antigen binding of the CAR constructs was characterized by staining with a HLA-A*02:01 restricted NY-ESO-1$_{157-165}$ (SLLMWITQC) peptide-specific, PE conjugated tetramer (E-H).

3.2 Re-directed CD8$^+$ T cells lysed target cells and secreted IFNγ in an antigen-specific manner

To demonstrate functionality of re-directed T cells *in vitro,* europium release assays were performed to indicate specific lysis of target cells. Stable-transfected HLA-A*02:01-positive T2 cells expressing HLA-A*02:01 restricted NY-ESO-1 peptides 157-165 (T2-1B, figure 3-4 A) and 157-167 (T2-1A, figure 3-4 B) served as target cell lines. NY-ESO-1 peptides of different length were used to test for antigen specificity. There was significantly higher specific lysis of target cells when re-directed T cells were transduced with T1-CD28/CD3ζ CAR compared to T1-CD3ζ (figure 3-4 C). Furthermore, the myeloma cell line U266 was used to assess the ability of re-directed T cells to lyse cells endogenously expressing NY-ESO-1 in the context of HLA-A*02:01. Cell lysis was target antigen specific since only T1-CD28/CD3ζ and not BW431/26-CD28/CD3ζ recognized U266 cells (figure 3-4 E).

IFNγ secretion was assessed by ELISA to demonstrate specific cytokine release. Re-directed T cells expressing the T1-CAR with the CD28 signaling domain secreted significantly higher antigen-specific IFNγ. Moreover, T1-CD3ζ CAR expressing re-directed T cells did not secrete

IFNγ in an antigen specific manner (figure 3-4 D). Control re-directed T cells recognizing CEA revealed only background levels of cell lysis and IFNγ release.

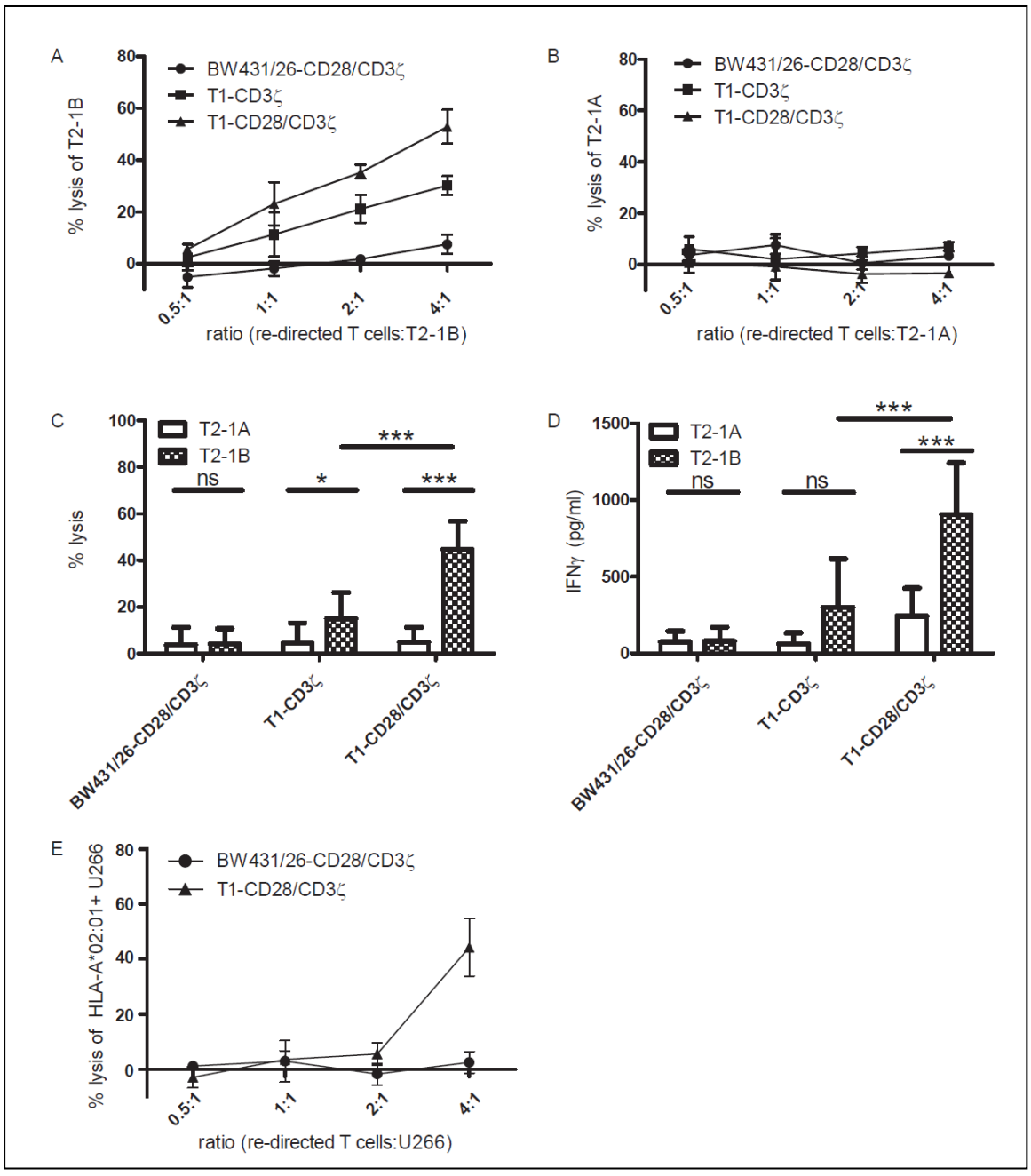

Figure 3-4: Representative experiment showing antigen-specific cytolysis of CAR re-directed CD8$^+$ T cells.

To demonstrate antigen specific cell lysis, re-directed T cells recognizing CEA (BW431/26-CD28/CD3ζ) and NY-ESO-$1_{157-165}$ peptide with (T1-CD28/CD3ζ) and without CD28 co-stimulatory domain (T1-CD3ζ) were co-cultured with stable-transfected T2 cells expressing HLA-A*02:01 restricted NY-ESO-1 peptides 157-165 (A) and 157-167 (B) at different effector to target ratios. Panel C depicts lysis of target cells at an effector: target ratio of 4:1 over 10 pooled experiments. To further show antigen specific cytokine secretion supernatants of co-cultivated cells were analyzed for IFNγ. Panel D depicts IFN γ release after 24 h of co-cultivation at an effector: target ratio of 4:1 over 10 pooled experiments. Re-directed T cells recognizing CEA (BW431/26-CD28/CD3ζ) and NY-ESO-$1_{157-165}$

peptide (T1-CD28/CD3ζ) were cultured with the HLA-A*02:01 positive and endogenously NY-ESO-1 expressing myeloma cell line U266 at different effector to target ratios (E). ***p<0.001, *p<0.05

3.3 CAR transduced T cells included subpopulations with effector and memory phenotype

Since expression of the two CARs resulted in immediate antigen specific functionality, CAR transduced T cells were phenotypically analyzed and expected to consist of subpopulations with effector T cells (T_{EFF}). Therefore, 6-color flow cytometry analysis (anti-hIgG, anti-CCR7, anti-CD57, anti-CD45RA, anti-CD28, anti-CD27) was employed to delineate sup-populations of re-directed T cells (hIgG positive cells) as depicted in figure 3-5.

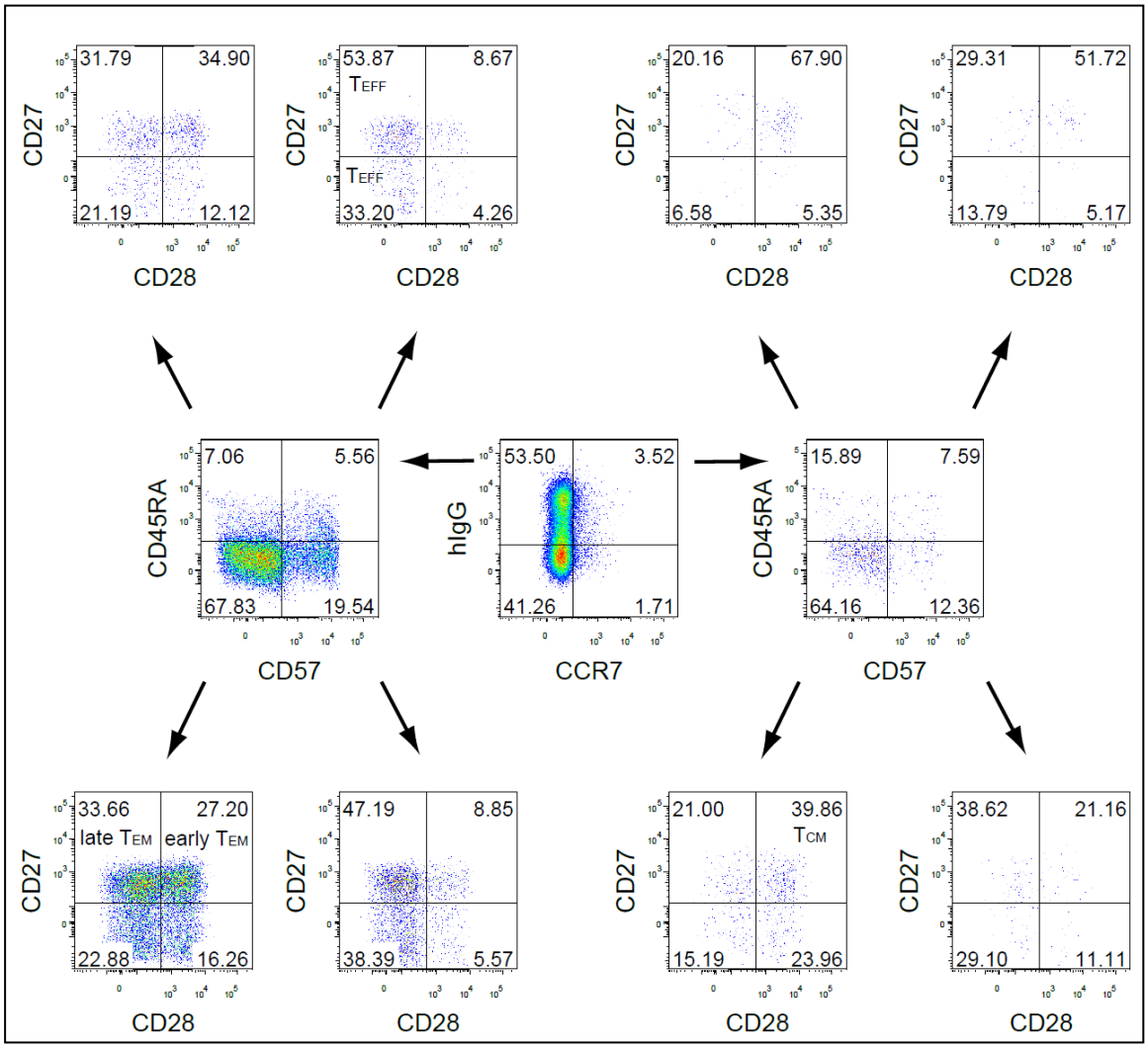

Figure 3-5: Representative phenotypical characterization of $CD8^+$ T cells expressing the T1-CD28/CD3ζ CAR by multi-parametric flow cytometry.

Pre-gated $hIgG^+CCR7^+$ or $hIgG^+CCR7^-$ T cells were arrayed in a CD45RA vs. CD57 2-parameter dot-plot. Cells in each of the four quadrants were then further subdivided by gating through CD27/CD28 dot-plots.

T cells from eleven consecutive donors were analyzed three days after transduction. Re-directed T cells included a $CCR7^-$ population which could be separated into subpopulations with T_{EFF} phenotype ($CCR7^-CD45RA^+CD57^+CD27^{+/-}CD28^-$) and T_{EM} phenotype ($CCR7^-CD45RA^-CD57^-CD27^+CD28^{+/-}$) (Petrausch et al., 2006). Furthermore, the $CCR7^+$ population included cells with a T_{CM} phenotype ($CCR7^+CD45RA^-CD57^-CD27^+CD28^+$) (figure 3-6 A). Comparison of all 11 donors showed no significant overall difference between both CARs (with and without CD28 signaling domain). However, transduction of the different CAR constructs resulted in a statistically significant different composition of single sub-phenotypes which included T_{EMRA}, late and early T_{EM} and T_{EFF}. Interestingly, phenotypical comparison of re-directed and non-transduced T cells revealed the same T cell distribution pattern (figure 3-6 B).

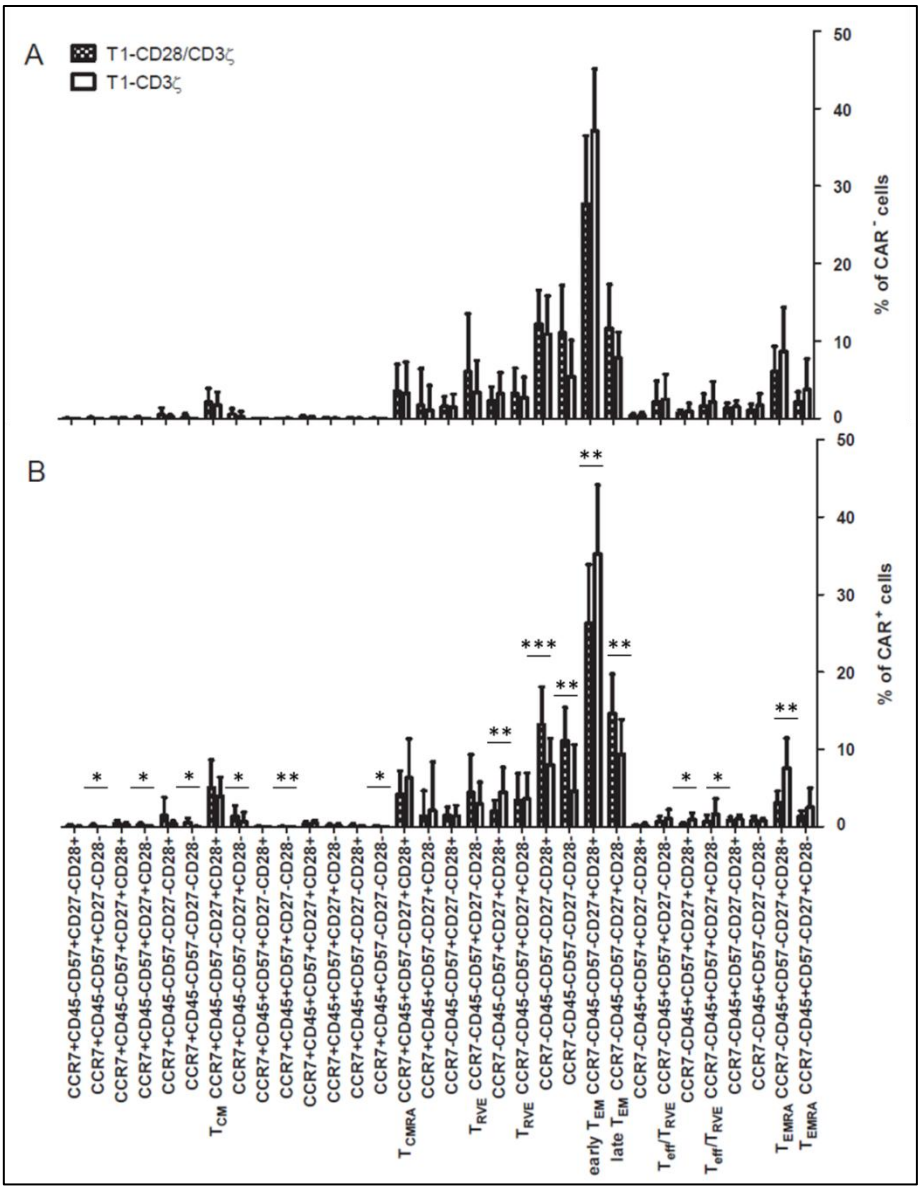

Figure 3-6 : Subpopulation signatures of transduced $hIgG^+CD8^+$ T cells (A) and non-transduced $hIgG^-CD8^+$ T cells (B) pooled over 11 individual donors 3 days after retroviral transduction.

The resulting 32 subpopulations of hIgG$^+$CD8$^+$ T cells were displayed on the X axis and were divided into 16 CCR7$^+$ and 16 CCR7$^-$ subpopulations. Subpopulations signatures were based on the combined positive and negative staining patterns for CD45RA/CD57/CD27/CD28 monoclonal antibodies within each CCR7 compartment. Each CCR7 staining compartment was subdivided into CD45RA$^-$ and CD45RA$^+$ subsets, and each CD45RA subpopulation was then further subdivided into CD57$^-$ and CD57$^+$ T cells. Final delineation of subpopulations was defined by expression of CD27 and CD28 on T cells in each subpopulation. T_{CM} = central memory T cell, T_{EM} = effector memory T cells, T_{EFF} = effector T cells, T_{EMRA} = effector memory T cell re-expressing CD45RA, and T_{RVE} = resting vigilant effector T cells ***p<0.001, **p<0.01, *p<0.05

3.4 CCR7$^-$ re-directed T cells secreted IFNγ and IL-2

Since re-directed T cells displayed a CCR7$^-$ phenotype which suggested the presence of effector and effector memory T cells, it was further hypothesized that re-stimulation of the CCR7$^-$ subset would result in the release of distinct cytokines. Therefore, re-directed T cells from six consecutive donors were activated with the anti-idiotypic (AI) Fab or control Fab, respectively. Activation of the CCR7$^-$ hIgG$^+$ subset resulted in antigen-specific release of IFNγ or IL-2 or the combination of both (figure 3-6 A and 3-6 B). Given the low cell count of CCR7$^+$ hIgG$^+$ T cells, no conclusion of their cytokine profile could be drawn.

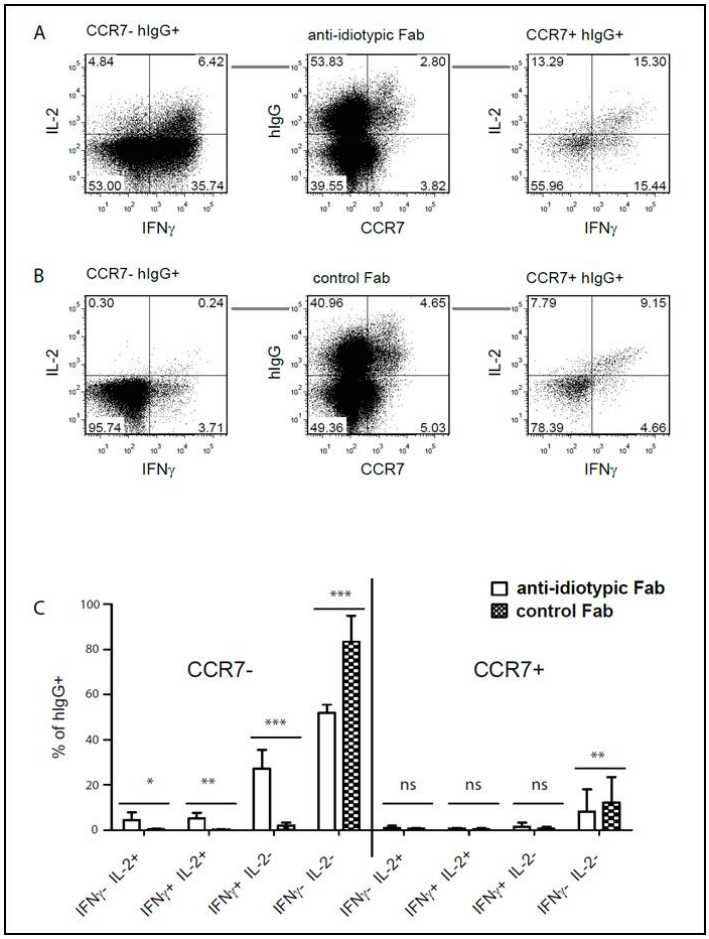

Figure 3-7: Intracellular analyses of re-directed T cells harboring the T1-CD28/CD3ζ CAR were conducted by flow cytometry three days after transduction.

Re-directed T cells were stimulated for 12 h with the anti-idiotypic antibody (A) and control Fab (B). hIgG$^+$ cells were gated in CCR7$^-$ and CCR7$^+$ cells. Both CCR7 compartments were arrayed in IL-2 vs. IFNγ dot-plots. Nine consecutive re-stimulations from individual donors were summarized in C. ***p<0.001, **p<0.01, *p<0.05, ns = not significant

3.5 CCR7$^+$ hIgG$^+$ T cells down-regulated CCR7 and expressed IL-2 after re-stimulation

Re-directed central memory T cells were detected by employing multi-color flow cytometry analysis (figure 3-6). Their antigen-specific functionality was tested and it was speculated that activation of re-directed CCR7$^+$ T cells resulted in differentiation towards CCR7$^-$ T_{EFF} and T_{EM} cells. Therefore, re-directed CCR7$^+$ T cells were sorted and re-stimulated with anti-idiotypic Fab AI or control Fab. Activation of the re-directed CCR7$^+$ T cell population resulted in a significant, antigen-specific reduction of hIgG$^+$ CCR7$^+$ T cells and a significant increase of hIgG$^+$ CCR7$^-$ T cells. In contrast, sorted re-directed CCR7$^-$ T cells showed no significant change of CCR7 expression (figure 3-8 A) suggesting no phenotypical change after activation. To demonstrate antigen-specific secretion of the memory signature cytokine IL-2, re-directed CCR7$^+$ T cells were sorted and re-stimulated. Re-directed CCR7$^+$ T cells demonstrated antigen-specific release of IL-2 and only low levels of IFNγ. In contrast, sorted re-directed CCR7$^-$ T cells produced primarily IFNγ and additionally IL-2. However, IL-2 secretion was significantly lower compared to CCR7$^+$ sorted re-directed T cells (figure 3-8 B).

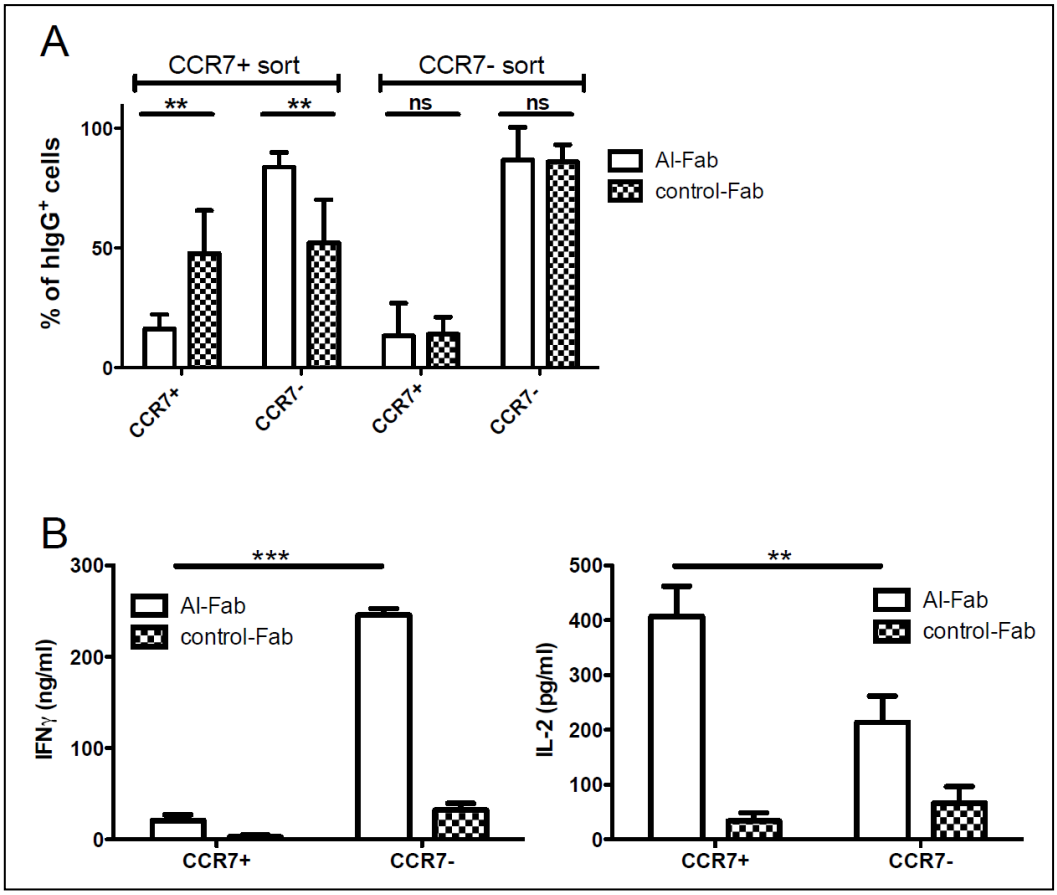

Figure 3-8: Sorting of hIgG$^+$CCR7$^+$ and hIgG$^+$CCR7$^-$ T cells and subsequent re-stimulation with anti-idiotypic antibody and control Fab.

CCR7 expression was re-assessed 10 days after re-stimulation in the presence of 50 ng/ml IL-15 for both, $hIgG^{+}CCR7^{+}$ and $hIgG^{+}CCR7^{-}$ sorted T cells in six individual experiments (A). IL-2 and IFNγ secretion from $hIgG^{+}CCR7^{+}$ and $hIgG^{+}CCR7^{-}$ sorted cells was measured by ELISA after 24 h of re-stimulation (B). ***p<0.001, **p<0.01, ns = not significant

3.6 Re-directed T cells protected against tumor outgrowth in a multiple myeloma xenograft model

The above findings indicated antigen-specific functionality of NY-ESO-1 specific re-directed T cells *in vitro*. A xenograft model in NOD/SCID/γ_c^{null} (NSG) mice was established to test whether these re-directed T cells were protective against a HLA-A*02:01/ NY-ESO-1 positive human myeloma cell line (U266).

3.6.1 Intravenous injection of U266 cells established a human multiple myeloma model in NOD/SCID/γ_c^{null} (NSG) mice

To establish a multiple myeloma xenograft model, NOD/SCID/γ_c^{null} mice were irradiated and subsequently intravenously or intraperitoneally injected with different doses of U266 cells to allow for tumor growth in the bone marrow or in the peritoneal cavity, respectively. U266 cells secrete the paraprotein hIgE which was measured in serum samples of animals and was used as a surrogate marker for tumor burden. hIgE kinetics of intravenously injected mice with the doses $6*10^6$, 10^7 and $2*10^7$ varied only marginally. Between day 25 and day 33 after i.v. injection, all mice (9/9) had measureable hIgE proteins in their blood which was followed by a rapid increase of hIgE levels over time. 2/3 mice i.p. injected with $2*10^7$ U266 cells showed first measurable hIgE levels at day 19, 1/3 mice only developed measureable hIgE levels 75 days after injection (figure 3-9).

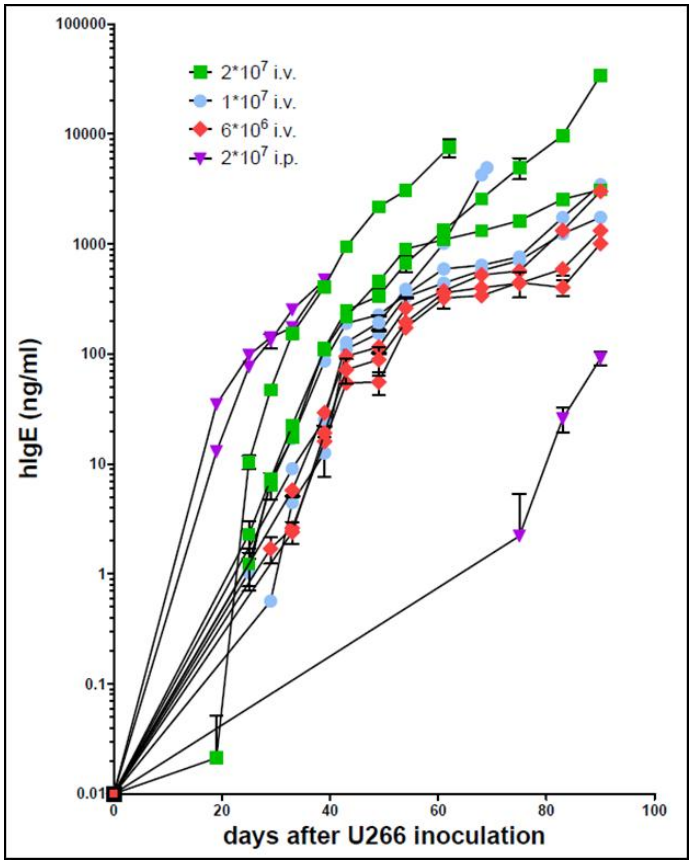

Figure 3-9: Xenograft model of injected U226 HLA-A*02:01/NY-ESO-1 positive myeloma cells.

After injection of different doses of U266 and different routes the concentration of secreted human IgE was measured by ELISA.

2/3 i.p. injected mice developed a solid tumor at the peritoneum of the size 7x8 mm at day 33 (figure 3-10 A). Mice showed no symptoms. No U266 cells were found in the blood or the bone marrow (data not shown). Mice i.v. injected with U266 cells developed with time (after day 65) disease symptoms, including ruffled fur, hunched posture, failure to eat and drink and hind limb paralysis. Mice with severe hind limb paralysis were X-rayed to detect osteolytic lesions (figure 3-10 B).

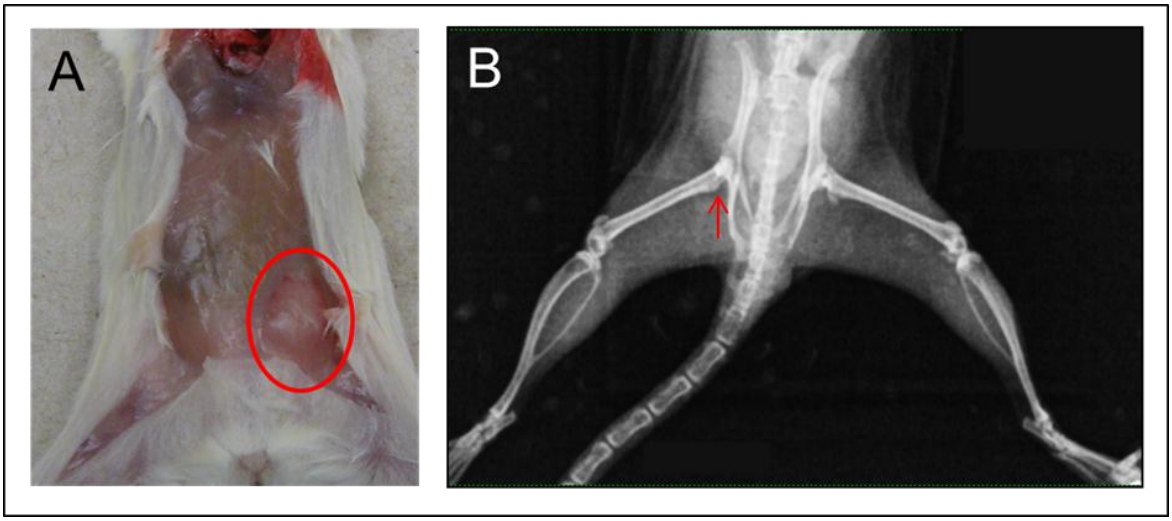

Figure 3-10: Growth of i.p. injected U266 cells at day 39 (A) and X-ray analysis of i.v. injected mice at day 69 (B).

Growth of a 7x8 mm sized tumor (red circle) was seen at the peritoneum (2/3 mice) after i.p. injection of $2*10^7$ U266 cells (A). X-ray of a mouse which suffered from severe hind leg paralysis indicated osteolytic lesions (red arrow, B).

Staining and subsequent flow cytometry analysis of HLA class I molecules were used to detect U266 cells. Bone marrow analysis of mice exhibiting severe hind limb paralysis showed a high percentage of HLA class I positive cells. This was found in the bone marrow of the spine as well as femur and tibia. In contrast, no HLA class I staining was detected in the blood (figure 3-11).

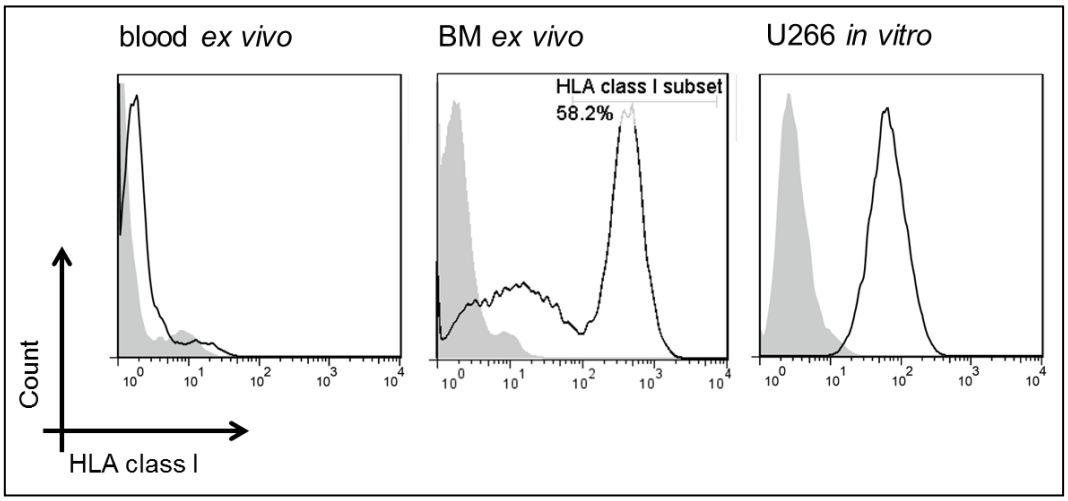

Figure 3-11: Flow cytometry analyses of the blood and bone marrow of U266 i.v. injected mice at day 69. The grey filled area shows the isotype control, the solid black line depicts HLA class I staining which was used to recognize U266 cells. As positive control served *in vitro* cultured U266 cells.

Furthermore, histopathological analyses confirmed massive U266 infiltration of the bone marrow of the femur (figure 3-12 A and B). Additionally, U266 cells invaded into the spinal cavity (figure 3-12 C and D). On day 69 after U266 injection, multiple myeloma cells were replacing the bone marrow environment and areas with normal residual hematopoiesis could be rarely detected (figure 3-12 E and F).

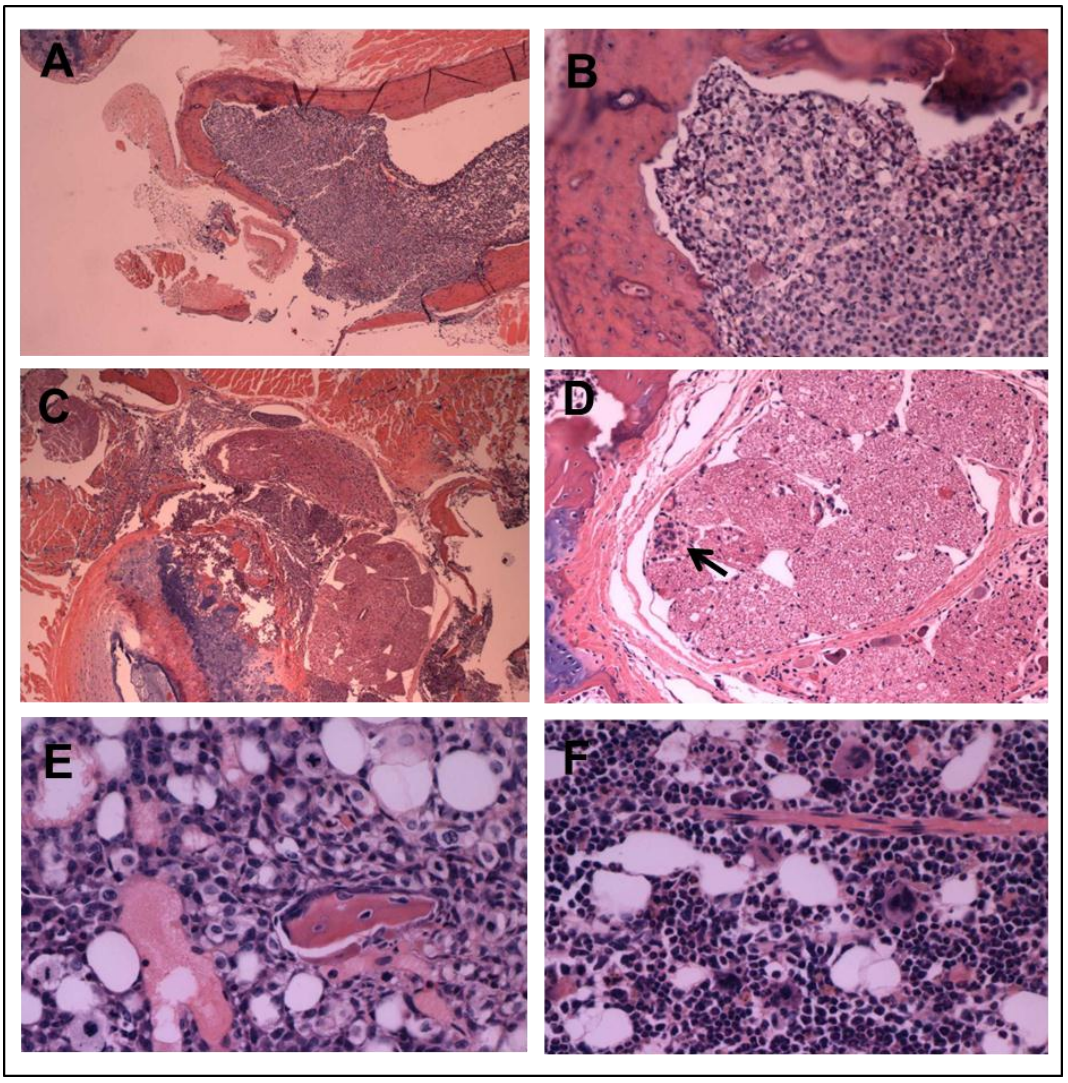

Figure 3-12: H&E stainings of femur (A, B) and spine (C, D) of NSG mice injected with 10^7 U266 cells at day 69 after injection.

Extensive accumulation of U266 cells was found in the femur of mice (A, B). U266 cells grew in the spinal cord (C) and formed nests (D, arrow). The junction between normal hematopoiesis and U266 outgrowth was shown in E and normal residual hematopoiesis (both bone marrow of the tibia) in F.

3.6.2 Protective capacity of re-directed T cells against multiple myeloma outgrowth

To investigate the protective capacity of re-directed T cells, 10^6 re-directed $CD8^+$ T cells specific for NY-ESO-$1_{157-165}$/HLA-A*02:01 complexes (T1-CD28/CD3ζ) or for CEA (BW431/26-CD28/CD3ζ) were intravenously injected 5 days after U266 transplantation. One group did not receive any treatment. Human IgE serum levels were measured by ELISA as a surrogate marker for tumor growth. No difference in hIgE levels between the three groups was observed (figure 3-13).

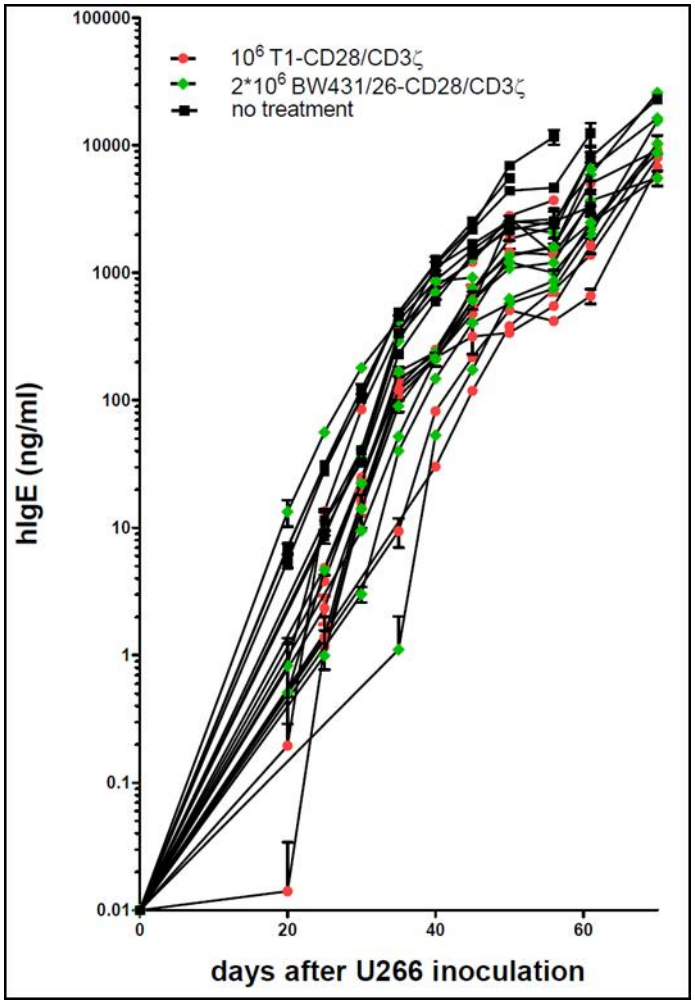

Figure 3-13: Xenograft model of i.v. injected U266 cells treated with re-directed T cells or left untreated.

Re-directed T cells (10^6 NY-ESO-1 specific re-directed T cells (●, red), $2*10^6$ CEA specific T cells (♦, green)) were injected at day 5 after U266 implantation or left untreated (■, black). The transduction efficiency of NY-ESO-1 specific re-directed T cells was 50 %. As specificity control served CEA-specific re-directed T cells.

The treatment schedule was changed and the numbers of injected re-directed T cells were raised as no delay in tumor outgrowth was observed when injected with 10^6 NY-ESO-1 specific T cells. 10^7 re-directed $CD8^+$ T cells were injected 5 days after tumor transplantation. T1-CD28/CD3ζ re-directed T cells protected mice against tumor outgrowth which was assessed by the surrogate marker hIgE (figure 3-14).

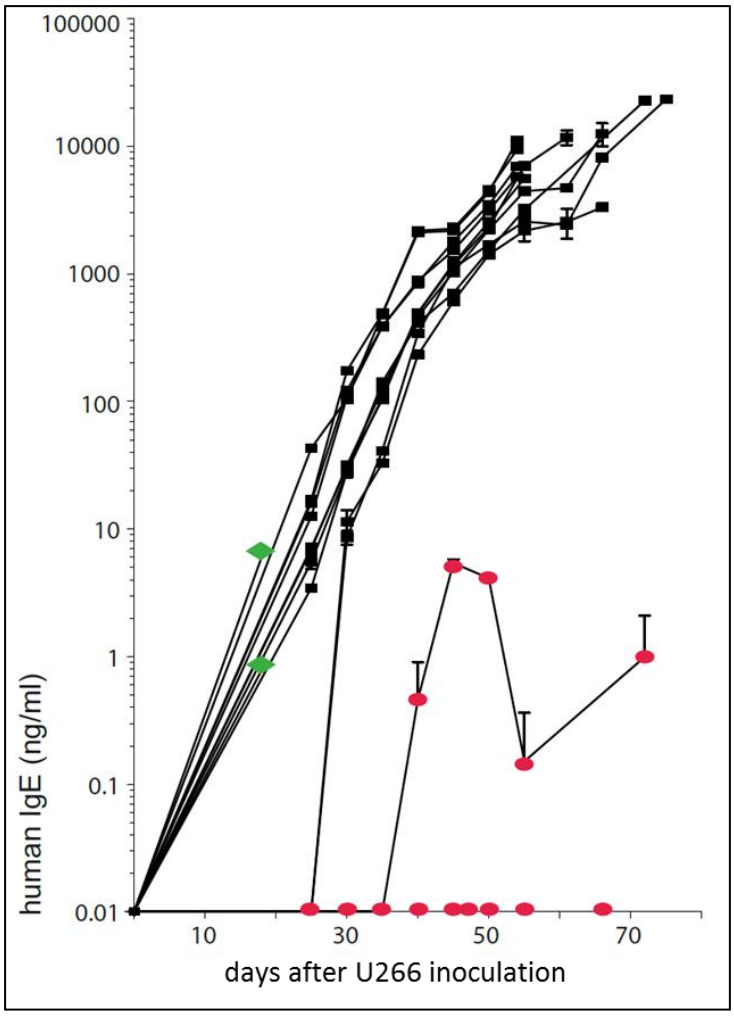

Figure 3-14: Xenograft model of intravenously injected U226 HLA-A*02:01/NY-ESO-1 positive myeloma cells.

Mice were treated with 10^7 NY-ESO-1 specific (●, red) or $2*10^7$ CEA specific (♦, green) or no (■, black) re-directed T cells. The transduction efficiency for re-directed NY-ESO-1 specific T cells was 50 %.

Human IgE levels were significantly different at various time points between the non-treated group and the T1-CD28/CD3ζ treated group as calculated by Mann-Whitney U-test indicating protection against tumor outgrowth in the bone marrow (table 3-1).

day	P-value (Mann-Whitney U-test)
25	0.000751
30	0.000064
35	0.000064
40	0.000300
45	0.000300
50	0.000579
55	0.006736
66	0.063603

Table 3-1: Mann-Whitney U-test showed statistical significant differences between untreated and T1-CD28/CD3ζ treated group up to day 55.

Mice treated with BW431/26-CD28/CD3ζ CAR positive T cells developed measurable IgE levels but succumbed early after CAR T cell injection. Macroscopic pictures showed massive destruction of the GIT (figure 3-15 A). However, microscopic H&E stainings of the lamina propria and the villi failed to show lymphocyte infiltrates (figure 3-15 B).

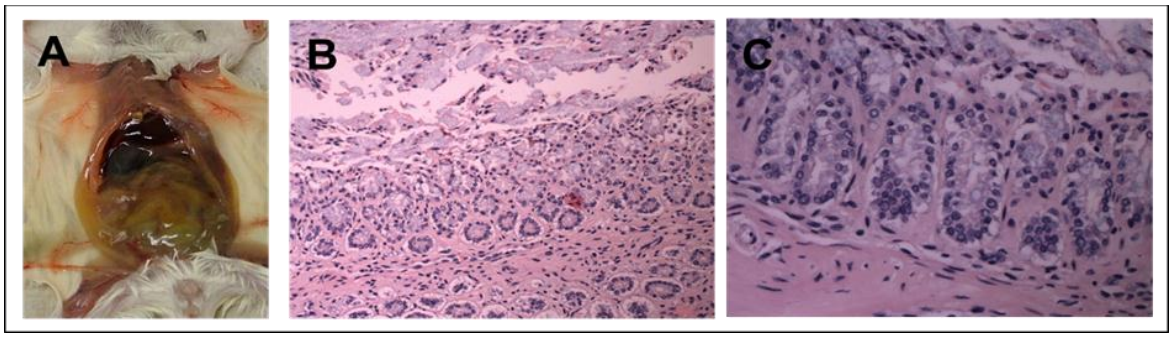

Figure 3-15: Macroscopic (A) and microscopic H&E (B, C) analyses of mice after injection of CEA-specific re-directed T cells.

Panel A depicts the atypical GIT of an injected mouse and panels B and are H&E stainings of the intestinal tract.

Mice were analyzed for hIgG$^+$ cells at various time points after transfer. Analysis of peripheral blood mononuclear cells revealed persistence of hIgG$^+$ CD8$^+$ T cells in 4 out of 10 mice until day 30 after adoptive transfer (figure 3-16). Re-directed T cells harbored an exclusive CCR7$^-$ phenotype and comprised between 6.5 and 1.4 % of total CD8$^+$ T cells.

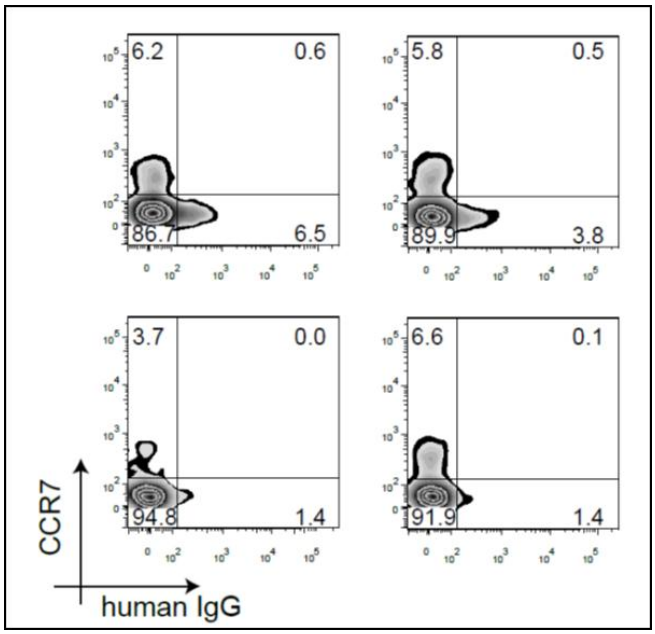

Figure 3-16: Re-directed T cells were found up to 30 days after transfer in the peripheral blood of different mice.

Representative dot plots of four individual mice are shown. Plots are shown for pre-gated human CD3$^+$CD8$^+$ cells.

After day thirty, 90% of the mice started to develop graft versus host disease (GvHD) like symptoms. They lost weight, displayed limited mobility, the fur was ruffled and the general appearance of mice was disrupted. The GIT track of mice with GvHD like symptoms appeared to be black (figure 3-17 A) and H&E sections of lung tissue revealed mixed inflammatory lymphocyte infiltrates with a high proliferation rate (figure 3-17 B). Liver

sections showed massive lymphocyte infiltration and proliferation. Extensive degradation of collagen and fibronectin as well as the destruction of hepatocytes could be observed in liver tissue (figure 3-17 C). However, the GIT was deprived of lymphocyte infiltrates and appeared to be normal (figure 3-17 D).

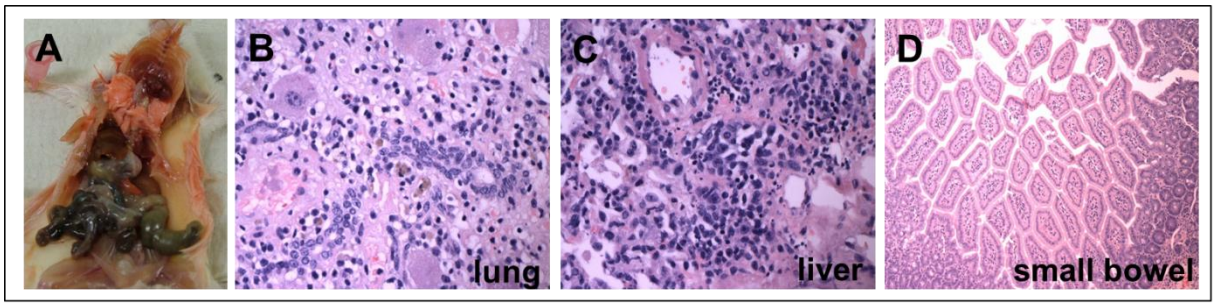

Figure 3-17: Macroscopic (A) and microscopic H&E (B-D) analyses of mice displaying GvHD-like symptoms.

Panel A shows the intestinal tract which appeared to be black. The H&E section of lung tissue (B) displayed mixed inflammatory lymphocyte infiltrates which had a high proliferative index. A high expansion of lymphocytes was seen in the liver section (C). Extensive fibrosis and the destruction of hepatocytes suggested GvHD-like disease. The small bowel (D) section was not infiltrated by lymphocytes and appeared normal.

Flow cytometry analyses of spleen, bone marrow and blood revealed a HLA class I/CD3 double positive population which was negative for hIgG (figure 3-18). The bone marrow and the spleen harbored up to 92 % and 65 % of $CD3^+$ cells, respectively.

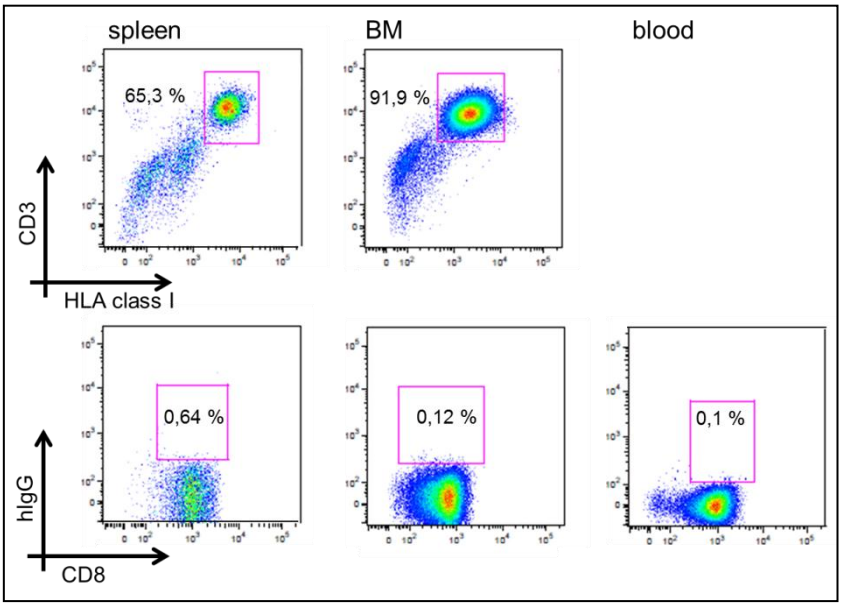

Figure 3-18: Flow cytometry analyses of mice displaying GvHD-like disease.

65 % of splenocytes and 92 % of bone marrow associated cells were CD3 and HLA class I double positive. This population also expressed CD8 but was negative for hIgG. In the blood, an extensive amount of $CD8^+$ cells was found which were $hIgG^-$.

4 Discussion

The concept of adoptive T cell transfer is gaining significant attention as phase I/II clinical trials (June, 2007) have demonstrated clinical responses (Rosenberg and Dudley, 2009) in patients with cancer of advanced stage. Most recently, the successful treatment of patients with chronic lymphoid leukemia has been reported (Porter et al., 2011). This project demonstrated antigen-specific functionality of HLA-A*02:01/ NY-ESO-$1_{157-165}$ re-directed T cells *in vitro*. Transduced T cells consisted of multiple subpopulations. The majority of $CD8^+$ re-directed T cells displayed an effector-memory phenotype. Interestingly, a subset of T cells with central memory phenotype was also antigen-specific. Furthermore, the data suggested a protective effect of adoptively transferred NY-ESO-1 specific re-directed T cells against neoplastic plasma cells in a xenograft model.

First and second generation CAR contructs were generated recognizing the HLA-A*02:01/ NY-ESO-$1_{157-165}$ complex and were used to retrovirally transduce $CD8^+$ T cells. The intracellular moiety consisted either of a CD3ζ only or a combined CD28/CD3ζ signaling domain. Both constructs achieved similar transduction efficacies and were able to recognize the target antigen in a specific manner (figure 3-3 and 3-4). However, functional analyses of the CARs revealed that additional CD28 signaling resulted in significantly increased target cell lysis and IFNγ release. The data are in line with data from Willemsen et al. (2005), who previously reported that activation of a HLA class I restricted CAR resulted in an increased lytic capability and cytokine secretion. In contrast, classic CARs that bind to cell surface molecules do not display elevated cytolysis but show increased IFNγ release (Hombach et al., 2001; Pinthus et al., 2003). Furthermore, it was demonstrated that cytolysis and cytokine release are highly antigen specific events. CARs did not recognize the HLA-A*02:01/NY-ESO-$1_{157-167}$ complex which only differs by two additional amino acids. This is in contrast to the previous report of an affinity maturated MAGE-A1/HLA class I restricted second generation CAR construct. Testing of cells transduced with this construct resulted in unspecific cytolysis and IFNγ release of HLA class I target cells which did not express the MAGE-A1 peptide (Willemsen et al., 2005). HLA-A*02:01 positive multiple myeloma cells with endogenous NY-ESO-1 expression were lysed at a target: effector ratio of 4:1. Cell lines stable transfected with the NY-ESO-$1_{157-165}$ peptide already showed antigen specific lysis at a target: effector ratio of 1:1. This observation can be attributed to the lower expression of antigen in endogenously NY-ESO-1 expressing cells.

Additionally, flow cytometry was employed to delineate subpopulations of CAR transduced T cells. Since a physiological and effective immune response results in multiple functionally different subsets of T cells including memory and effector cells (Sallusto et al., 2004), it is

important to have a similar composition of re-directed T cell subsets. Such multi-functionality is of special interest when immune therapy is intended to control minimal tumor burden over time. $CD8^+$ T cells can be classified in naïve, effector T cells (T_{EFF}) and memory T cells (T_M) depending on their antigen-experience, activation state, longevity and proliferation capacity (Sallusto et al., 2004). Even if the lineage development of $CD8^+$ T_M cells is not finally understood, different subpopulations of $CD8^+$ T cells are phenotypically and functionally characterized. Central memory T cells (T_{CM}) are phenotypically described as $CCR7^+CD45RA^-CD57^-CD27^+CD28^+$ whereas effector memory T cells (T_{EM}) have the $CCR7^-CD45RA^-CD57^-CD27^+CD28^{+/-}$ phenotype (van Baarle et al., 2002).

Circulating or tissue-resident $CD8^+$ T_{EM} display immediate effector function upon antigen recognition. T_{CM} reside in the T cell areas of lymphoid tissues where they can mount a rapid recall response after recognizing their cognate antigen presented by dendritic cells (Wherry et al., 2003). Multiple effector and memory populations within the CAR re-directed T cells were phenotypically identified based on consensus models of 3 to 7 marker analyses (Ahmed et al., 2009; Klebanoff et al., 2006; Kern et al., 1999; Geginat et al., 2003; Walker et al., 2008). Primarily early and late effector memory phenotypes were found in the re-directed T cells ($CCR7^-CD45RA^-CD57^-CD27^+CD28^+$ and $CCR7^-CD45RA^-CD57^-CD27^+CD28^-$, respectively) and small populations of fully differentiated effector T cells ($CCR7^-CD45RA^+CD57^+CD27^{+/-}CD28^-$). This was expected as the re-directed T cell populations showed immediate IFNγ secretion and cytotoxicity. Since the majority of re-directed T cells had a late and early T_{EM} phenotype, the immediate antigen-specific response *in vitro* and the protection *in vivo* were presumably mediated by T_{EM} cells. Unexpectedly, a minor population of central memory re-directed T cells ($CCR7^+CD45RA^-CD57^-CD27^+CD28^+$) was detected. Barber et al. (2008) showed an early effector differentiation phenotype of chimeric NKG2D expressing $CD8^+$ T cells after 10 days of culture. Neeson et al. (2010) reported that Le^Y re-directed T cells mainly displayed effector and central memory phenotypes by the end of the transduction. These differences may result from the different T cell activation and retroviral transduction protocols. In both publications, T cells were stimulated by anti-CD3 (OKT3) and IL-2 for 3 days and subsequently retrovirally transduced for 5 or 7 days, respectively. In contrast, in this thesis T cells were activated with anti-CD3, anti-CD28 monoclonal antibodies and IL-2 for two days and the transduction lasted for 4 days via 293T co-culture. Neeson et al. analyzed a second generation CAR construct harboring a CD28/CD3ζ domain and Barber et al. a primary construct expressing the CD3ζ chain only. Here, analysis of 11 different donors revealed only slight differences in the phenotypical composition of re-directed T cells expressing different signaling domains. Most notably, re-directed T cells without CD28

signaling displayed higher amounts of early T_{EM}, less late T_{EM} cells and more T_{EMRA} cells compared to CD28/CD3ζ re-directed T cells.

A minor population of central memory re-directed T cells ($CCR7^+CD45RA^-CD57^-CD27^+CD28^+$) was detected comprising 3 - 8 % of the re-directed T cell pool. These cells are of special interest as they might be able to generate functional effector T cells after antigen re-encounter and thus be able to control residual dormant tumor cells. Kalos et al. (2011) reported the infusion of αCD19-41BBζ CAR transduced $CD3^+CD28^+$ T cells from three patients with advanced chronic lymphocytic leukemia. 56 days after adoptive T cell transfer, the CAR^+ $CD8^+$ T cell compartment displayed primarily an effector phenotype ($CCR7^-CD27^-CD28^-$). However, on day 169 a population evolved with features of a central memory phenotype ($CCR7^+CD27^+CD28^+CD57^-$). These engineered T cells expanded >1000 fold in patients and travelled to the bone marrow, resulting in a complete remission in two of three patients (Kalos et al., 2011). Furthermore, alternative approaches to generate a functional and persisting CAR positive memory pool are currently elucidated. Pule et al. (2008) described an approach to express CARs on CTLs specific for viral antigens. These CTLs were supposed to display a memory phenotype and to receive superior co-stimulation *in vivo* by engaging with APCs through their native TCR. A first generation CAR was generated recognizing the GD2 antigen expressed by neuroblastoma tumor cells. CAR positive CTLs survived on a higher level over the 6-week study period compared to bulk transduced T cells (Pule et al., 2008). However, after the 6-week period CAR positive CTLs became undetectable. Nevertheless, the follow-up study showed three out of eleven patients with active disease achieved complete remission and persistence of CAR CTLs was concordant with co-infusion of $CD4^+$ T cells and $CD45RO^+CD62L^+$ central memory cells (Louis et al., 2012). Terakura et al. (2012) reported a method to generate second generation CAR specific $CD8^+$ T cells derived from virus-specific central memory cells. An elaborate method was employed to obtain virus-specific T cells from $CD45RA^-CD62L^+CD8^+$ central memory T cells from donor blood. These cells were able to expand *in vitro* and form a large pool of CAR positive effector T cells exhibiting target-antigen specific effector functions in vitro (Terakura et al., 2012).

Re-directed T cells with CD28/CD3ζ signaling domain were functionally superior when compared to ones with the CD3ζ domain only. Therefore, T cells harboring the CD28 signaling domain were analyzed in more detail to investigate functional differences of the phenotypically delineated T cell subpopulations *in vitro*. Since phenotypical analyses suggested antigen-specific functionality was mainly mediated by T_{EM}, the cytokine profile of the $CCR7^-$ re-directed subpopulation was studied. Re-stimulation of $CCR7^-$ re-directed T cells resulted in the simultaneous release of IFNγ and IL-2 in a portion of transduced T cells.

This cytokine profile supports our phenotypical data. Early effector memory T cells produce IFNγ and IL-2 after antigen recognition (Cui and Kaech, 2010), which represent a substantial subset of the $CCR7^-$ re-directed T cells. Furthermore, sorting and subsequent analysis of cytokine secretion of $CCR7^+$ and $CCR7^-$ re-directed T cells revealed that $hIgG^+$ $CCR7^+$ T cells released significantly more IL-2 than $CCR7^-$ re-directed T cells. $CCR7^-$ CAR^+ T cells released more IFNγ than $CCR7^+$ CAR^+ T cells, and they also secreted IL-2. This is in accordance with previously reported data which demonstrated that $CD45RA^-$ $CCR7^-$ T_{EM} cells secrete high amounts of IFNγ and, to a lesser extent, IL-2 whereas $CD45RA^-$ $CCR7^+$ T cells released mainly IL-2, the signature cytokine of central memory cells (Sallusto et al., 1999).

Although memory lineage development is still controversial (Ahmed et al., 2009), data suggest that secondary activation of T_{CM} results in strong proliferation and differentiation into T_{EFF} (Sallusto et al., 1999). In our model, re-stimulation of $CCR7^+$ re-directed T cells led to down-regulation of CCR7 supporting differentiation of central memory into effector T cells.

To explore the *in vivo* functionality of re-directed T cells, a xenograft model was chosen which mimics the pathology of human multiple myeloma.

Multiple Myeloma (MM) is characterized by neoplastic transformed plasma cells in the bone marrow (Palumbo and Anderson et al., 2011) and, in general, considered to be an incurable disease. It accounts for about 1 % of all cancers and more than 10 % of blood borne malignancies. Multiple myeloma is an exclusive post-germinal center cancer, which is verified by the fact that throughout the clinical course the Ig gene sequences are somatically hypermutated and remain constant (Bakkus et al., 1992; Sahota et al., 1997). In recent years, many therapeutic advances have been made to treat multiple myeloma patients including combined chemotherapy and hematopoietic stem cell transfer but most patients cannot expect long-term remission. This is due to drug-resistant or minimal residual disease (Hideshima and Anderson et al., 2002). Additionally, the lack of *in vivo* systems which mimic the growth of human multiple myeloma cells and the development of clinical manifestations hampered further development of new therapeutic strategies. New approaches have to be developed for eradication or control of residual malignant plasma cell burden (Szmania et al., 2006). Therefore, a xenograft multiple myeloma model was established to evaluate the functionality of HLA-A*02:01/ NY-ESO-$1_{157-165}$ re-directed T cells *in vivo*.

Immunodeficient mice derived from the inbred mouse strain NOD/ShiLtJ which also lack the interleukin-2 receptor γ chain (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ; NOD scid gamma; NSG) served as animal model for human multiple myeloma. NSG mice lack B and T cells and show several defects of innate immunity, namely impaired dendritic cell function (no production of

IFNγ), a complete lack of NK cells, impaired production of interleukin-1α by macrophages, and an absent hemolytic complement system (Shultz et al., 1995).

Before establishment of the NSG mouse strain, *in vivo* human multiple myeloma models have been carried out with SCID and NOD/SCID mice. However, simple intravenous injection of multiple myeloma cells did not lead to engraftment (Yaccoby et al., 1998; Yaccoby and Epstein, 1999; Pilarski et al., 2000; Urashima et al., 1997). Miyakawa et al. (2003) established engraftment of the human multiple myeloma cell line U266 in NOG mice (also NOD scid IL2Rγ deficient mice) by sublethal irradiation with 2.4 Gy followed by intravenous injection of $2x10^6$ U266 cells. All mice developed disease symptoms such as hunched posture, rough fur, failure to eat and drink and, finally, severe hind leg paralysis around 6 weeks after tumor cell injection. Three different doses of U266 cells were injected i.v. into NSG mice after 2.4 Gy irradiation (figure 3-9). All mice developed disease symptoms and severe hind leg paralysis between week 6 and 10. Histological and flow analyses revealed engraftment of U266 cells only in the bone marrow and not in the blood (figure 3-11, 3-11) which is in accordance with Miyakawa et al. (2003). U266 cells specifically invaded the murine bone marrow which allowed the development of clinical manifestations seen in myeloma patients (Roodman 1997; Dhodapkar et al., 1998). Furthermore, on X-ray films the formation of osteolytic lesions of the trabecular bones near the spine were detected (figure 3-10 B). Histological studies of U266 transplanted NOG mice showed osteolytic lesions of cortical bones and loss of trabecular bones in the sternum and spine (Miyakawa et al., 2003). Additionally, U266 tumor growth was confirmed by measuring hIgE serum levels as surrogate marker. Continuous hIgE production was observed which increased over time (figure 3-9), likely due to proliferation and expansion of U266 cells in the murine bone marrow.

10^6 re-directed T cells were injected in the tail vein five days after i.v. transplantation of 10^7 U266 cells. No delay in tumor growth was observed between different treatment groups. Therefore, a new experimental outline with injection of 10^7 re-directed T cells was set. 9/10 mice treated with 10^7 T1-CD28/CD3ζ CAR positive T cells did not develop any detectable hIgE levels compared to the untreated control group. The statistical significance was confirmed applying a Mann-Whitney U-test up to day 55. Peinert et al. (2010) injected subcutaneously into NOD/SCID mice a deposit of RPMI 8226-13 cells, a Le^Y positive MM cell line. One day later they injected 10^7 retrovirally transduced anti-Le^Y-scFv-CD28/CD3ζ T cells. Peinert et al. (2010) reported a delay of plasmacytomas in the specifically treated group, resulting in an improved overall survival.

Mice treated with BW431/26-CD28/CD3ζ CAR positive T cells succumbed between day 7 and 16. However, these mice developed measurable hIgE levels at early time points

indicating the specificity of the re-directed T cells *in vivo* (figure 3-14) as seen *in vitro* (figure 3-4). This early death of mice and restriction to the CEA re-directed T cells injected group suggested an on-/off-target effect of the injected CEA-specific T cells than the induction of a xeno-GvHD reaction. Murine analogues of human CEA share up to 100 % of its sequence. They are glycoproteins expressed in the GIT of human and mice. Macroscopic analyses showed massive destruction of the GIT indicating on-target specificity of CEA specific re-directed T cells in the murine gut (figure 3-15).

Furthermore, NY-ESO-1 CAR transduced T cells were present in 40 % (4/10) of peripheral blood samples of treated animals after 30 days of adoptive transfer. Interestingly, hIgG expression did not coincide with CCR7 expression, indicating an effector phenotype of re-directed T cells. This finding is consistent with flow cytometry data showing mainly $CCR7^-$ phenotype of re-directed T cells (figure 3-5). Additionally, down-regulation of the CCR7 receptor was observed after antigen re-stimulation *in vitro* (figure 3-8). Zhao et al. also reported a gradual loss of CAR expression but was able to prolong persistence of re-directed T cells by adding a 4-1BB cytoplasmic domain to the CD28/CD3ζ moiety *in vivo* (Zhao et al., 2009). Most recently, the formation of central memory cells was detected in re-directed T cells with CAR consisting of a 4-1BB cytoplasmic domain and CD3ζ signaling domain after the transfer into patients with chronic lymphoid leukemia (Kalos et al., 2011).

After day 30, 90 % of mice injected with NY-ESO-1 specific T cells started to develop symptoms of xeno-GvHD, including weight loss, ruffled fur and limited mobility (Gregoire-Gauthier et al., 2011). It was speculated that irradiation of NSG mice carrying a *Prkdc* gene mutation led to irreparable tissue damage (Kirchgessner et al., 1995). This damaged and presumably inflamed tissue was then infiltrated by xeno-reactive T cells causing GvHD. Ito et al., (2009) reported death of NOG mice irradiated with 2.5 Gy and i.v. injected with 10^7 hPBMCs after day 14. This difference in time delay may be due to i.v. injection of human $CD8^+$ T cells only compared to whole hPBMCs. Further histological analyses (figure 3-17) revealed massive lymphocyte infiltration in lung and liver which is in accordance to Ito et al. (2009). Interestingly, the small bowel appeared microscopically to be physiological normal though the GIT was colored black which might be due to inner bleedings. In accordance, Rijn et al. (2003) and Ito et al. (2009) observed heavy lymphocyte infiltrations in lung and liver but not in the gut after induction of xeno-GvHD. Flow cytometry analyses of bone marrow, spleen and blood confirmed high levels of human $CD8^+$ T cells (figure 3-18). Ito et al., (2009) reported high ratios of human $CD45^+$ cells in spleen and blood but lower ratios in bone marrow (20 %-40 %). Around 90 % of cells found in the bone marrow stained for human $CD8^+$. This might be due to the initial re-direction of CAR transduced $CD8^+$ T cells towards U266 cells which homed to the murine bone marrow.

In summary, it was demonstrated that NY-ESO-$1_{157-165}$/HLA-A*02:01 re-directed T cells with a high affinity CAR exhibit strong effector functions without loss of specificity when CD28 signaling is provided through the CAR. Re-directed effector and central memory T cells were phenotypically identified which displayed characteristic antigen-specific functionality *in vitro*. Furthermore, CAR re-directed T cells were able to prevent tumor outgrowth of multiple myeloma in a xenograft model, which mimicked the development and pathogenesis of human MM. The results are a rational for the potential clinical use of NY-ESO-$1_{157-165}$/HLA-A*02:01 re-directed T cells for consolidation therapy in multiple myeloma. $CCR7^+$ re-directed T cells are of special interest for further development of adoptive T cell therapy to not only resolve existing primary tumors but also to keep residual or dormant tumor cells under control over long time. Approaches have to be developed to exploit the full therapeutic and protective potential of these already existing central memory T cells in the retrovirally transduced T cell population.

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7 Curriculum Vitae

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Education and experience

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Jul 2005 – Jan 2008	Technical University of Munich, Munich, Germany Master (Sc) course of studies `Molecular Biotechnology´
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Sep 2002 - Oct 2002	Internship at Bundesanstalt für Fleischforschung, Kulmbach, Germany Responsibilities: Real Time PCR

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Presentations

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